FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

THE ROLE OF GLIPTINS ON ADIPOGENESIS

Marta Maria Vieira Matutino Falcão Estrada

Dissertação elaborada com vista à obtenção do Grau de Mestre em Biotecnologia

Orientadores:

Professora Doutora Cláudia Cavadas

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Abstract

Dipeptidyl-peptidase IV (DPPIV) (E.C.3.4.14.5.) is a 110 KDa peptidase expressed in almost every tissue of the human body. DPPIV expression is altered in obese conditions. Preliminary studies in our laboratory showed that this enzyme stimulates pre-adipocyte differentiation and lipid accumulation using a murine pre-adipocyte cell line. Vildagliptin, sitagliptin and saxagliptin are a new class of DPPIV selective inhibitors used in diabetes to increase the half-life of some insulin-stimulating hormones. The aim of this study is to evaluate, the effect of gliptins on the adipose tissue formation (adipogenesis and lipolysis). Using a pre-adipocyte murine cell line, 3T3-L1 we analysed the effect of gliptins on lipid accumulation. Our results showed that gliptins reduced both basal and stimulated lipid accumulation. We further evaluated if gliptins could modulate lipolysis or adipogenesis. Our studies show that gliptins do not induce lipolysis but play an inhibitory role on adipogenesis. This inhibition is achieved by inhibiting the expression of a transcription factor crucial for adipocyte differentiation, PPARγ. It was also observed that gliptins inhibit lipid accumulation through PKA pathway.

Neuropeptide Y (NPY) is a DPPIV substrate that acts through six G-protein-coupled receptors: Y_1, Y_2, Y_3, Y_4, Y_5 and y_6. Previous studies in our lab showed that DPPIV stimulates lipid accumulation through cleavage of NPY_{1-36} into NPY_{3-36}. The cleaved peptide stimulates lipid accumulation through Y_2 receptor activation. The second aim of our study was to evaluate the effect of gliptins on NPY-stimulated lipid accumulation. Our results show that gliptins inhibit NPY-induced lipid accumulation. We also analysed the intracellular mechanism of action of NPY and conclude that NPY stimulates lipid accumulation through modulation of PKA.

The present study suggests that gliptins can be used as new putative pharmacological strategies to prevent adipose tissue increase without the risk of dyslipidemia.

Key words: Dipeptidyl-peptidase IV; Gliptin; Neuropeptide Y; Adipocyte; Protein Kinase A

Resumo

A Dipeptidyl-peptidase IV (DPPIV) (E.C.3.4.14.5.) é uma peptidase de 110 KDa expressa em quase todos os tecidos do corpo humano. A sua expressão encontra-se alterada em situações de obesidade. Estudos anteriores, realizados no nosso laboratório, demonstraram que a DPPIV estimula a diferenciação de adipócitos e a acumulação lipídica nos adipócitos. A vildagliptina, a sitagliptina e a saxagliptina fazem parte de uma nova classe de inibidores selectivos da DPPIV cuja função principal é aumentar o tempo de meia vida de algumas hormonas responsáveis pelo estímulo da produção de insulina. O objectivo principal deste trabalho consiste em avaliar o efeito que as gliptinas têm na formação do tecido adiposo (adipogénese e lipólise). Usando uma linha celular de pre-adipócitos de murganho (3T3-L1), foi testado o efeito das gliptinas na acumulação lipídica. Os resultados obtidos demonstram que as gliptinas reduzem tanto a acumulação lipídica basal como a acumulação lipídica induzida pela insulina. De seguida avaliámos se as gliptinas exerciam efeito na lipólise ou na adipogénese. Verificámos que apesar de as gliptinas não provocarem lipólise, têm um efeito inibitório na adipogénese. Esta inibição ocorre pela inibição da expressão de um factor de transcrição, PPARγ, crucial para a diferenciação dos adipócitos. Observámos ainda que as gliptinas inibem a acumulação lipídica via PKA.

O neuropéptido Y (NPY) é um conhecido substrato da DPPIV cuja acção ocorre pela activação de seis receptores acoplados à proteína G: Y_1, Y_2, Y_3, Y_4, Y_5 e y_6. Estudos anteriores realizados no nosso laboratório demonstraram que a DPPIV estimula a acumulação lipídica através da clivagem do NPY_{1-36} em NPY_{3-36}. O peptídeo clivado estimula a acumulação lipídica através da activação do receptor Y_2. O segundo objectivo deste trabalho consistiu na avaliação do efeito das gliptinas na acumulação lipídica induzida pelo NPY. Verificamos que as gliptinas reduzem esta acumulação lipídica induzida pelo NPY, e que este mecanismo era mediado pela acção da PKA.

O presente estudo sugere que os inibidores selectivos da DPPIV são possíveis estratégias farmacológicas para prevenir o aumento do tecido adiposo sem o risco de dislipidémia.

Palavras chave: Dipeptidil-peptidase IV; Gliptina; Neuropéptido Y; Adipócito; Proteína Cinase A

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Abbreviations

59-AMP	59-adenosine monophosphate
7TM	seven-transmembrane-helix
a2-AR	a2-adrenoreceptor
AC	Adenylate cyclase
ACS	acyl-coenzyme A synthetase
ADA	adenosine deaminase
ADRP	adipose differentiation-related protein
ALBP	Adipocyte Lipid binding protein
AMPK	AMP-activated protein kinase
aP2	adipocyte fatty acid binding protein
AR	adrenergic receptor
ATGL	adipocyte triglyceride lípase
AT	adipose tissue
ATP	adenosine triphosphate
β-AR	β -adrenoceptor
BAT	brown adipose tissue
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
cAMP	AMP cyclic
CPON	C-flanking peptide of NPY
CREB	cAMP-responsive element-binding protein
DEX	dexamethasone
DPPIV	Dipeptidyl-peptidase IV
ERK	extracellular signal-regulated kinase
FA	fatty acids
FABPm	fatty acid binding protein
FAT	fatty acid translocase
FATP	fatty acid transport protein
FBS	fetal bovine serum

FDA	food and Drug Administration
FFA	free fatty acid
GC	guanyl cylase
GHRF	growth-hormone releasing factor
Gi	inhibitory GTP-binding protein
GIP	gastric inhibitory peptide
GLP-1	glucagon-like peptide-1
GLUT4	glucose transporter type 4
glycerol 3-P	glycerol 3-phosphate
GRP	gastrin-releasing peptide
Gs	stimulatory GTP-binding protein
HSL	hormone-sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	half maximal inhibitory concentration
IL-6	Interleukin-6
IR	insulin receptor
IRS	insulin receptor substrate
LD	lipid droplets
LPL	lipoprotein lipase
MAPK	mitogen activated protein kinase
MCE	mitotic clonal expansion
MEF	mouse embryo fibroblast
MGL	monoglyceride lipase
MIG	monokine induced by gamma interferon
MIX	methylisobutylxanthine
MSC	mesenquimal stem cells
NK	natural killer
NPY	neuropeptide Y
PACAP	pituitary adenylate-cyclase-activating polypeptide
PBEF	pre-B-cell colony-enhanced factor

PBS	phosphate buffered saline
PDE-3B	phosphodiesterase 3B
PI3-K	phosphatidylinositol-3-phosphate kinase
PKA	protein kinase A
PKB	protein kinase B
PKG	protein kinase G
POMC	proopiomelanocortin
PP	pancreatic polypeptide
PPARγ	peroxisome proliferator-activated receptor γ
PYY	peptide YY
RBP 4	retinol-binding Protein 4
RXRα	retinoid receptor α
SEM	standard error of the mean
SP	substance P
T2DM	Type 2 Diabetes *Mellitus*
TAG	triglyceride molecules
TNF – α	tumor Necrosis Factor – α
TZDs	thiazolidinediones
UCP-1	uncoupling protein – 1
VIP	vasoactive intestinal peptide
VLDL	very low density lipoproteins
WAT	white adipose tissue
ZAP-70	zeta associated protein-tyrosine kinase of 70000MW

Chapter 1: Introduction

1.1 Adipose tissue

The adipose tissue (AT) is a mesodermal tissue composed by several types of cells [1, 2]. One third are mature adipocytes whereas the other two thirds are small blood vessels, nerve tissue, fibroblasts and pre-adipocytes [3]. In mammals, the adipose tissue is divided in two types of tissue: brown adipose tissue (BAT) and white adipose tissue (WAT), the former being responsible for energy dissipation in the form of heat, through non-shivering thermogenesis, and the later being involved in energy accumulation, mainly in the form of triglycerides [2, 4, 5].

Brown adipose tissue (BAT) contains predominantly adipocytes, which are rich in mitochondria and possess small multilocular lipid droplets (LD) [1, 6]. In humans, this tissue is present in neonates and new-born children, and is distributed through several areas of the body: pancreas, kidneys, adrenal glands, interscapular region (shoulder), muscles in the neck, in the axillae, trachea, esophagus and surrounding blood vessels [2]. Brown adipocytes main function is to transform energy from food into heat, through oxidative phosphorylation by uncoupling protein – 1 (UCP-1), present in the inner membrane of the mitochondria. In most of the tissues, where UCP-1 is absent, protons are pumped out of the mitochondrial matrix into the intermembrane space, generating an electrochemical gradient across the membrane. An ATPase would dissipate this gradient by pumping these protons back into the matrix, transforming ADP into ATP. However, when UCP-1 is present proton gradient is dissipated and ATP production is not allowed, thereby generating heat [1, 2, 4, 7, 8].

White adipose tissue (WAT) is found in several anatomically and physiologically distinct depots. There are two main white adipose tissues: the visceral and the subcutaneous adipose tissue. The first can be divided in omental adipose tissue, mesenteric adipose tissue and retroperitoneal adipose tissue [2]; whereas the second can be divided in superficial and deep subcutaneous adipose tissue [2]. WAT contains predominantly spherical white adipocytes that accumulate lipids, in the form of triglycerides, within one large lipid droplet [1, 2]. Besides accumulating triglycerides, the adipose tissue can also secrete molecules with endocrine, paracrine and autocrine functions [9].

1.2 Adipogenesis

Adipogenesis is a process that corresponds to pre-adipocytes proliferation and differentiation into mature adipocytes, see figure 1.1 [1]. Pre-adipocytes have origin in mesenquimal stem cells (MSC) which, besides adipocyte differentiation, are also capable of differentiating into osteoblasts, chondrocytes, myoblasts and connective tissue [1]. During the differentiation process that begins after birth, pre-adipocytes go through four different stages before becoming mature adipocytes: i) growth arrest, ii) clonal expansion, iii) early differentiation and iv) terminal differentiation [1]. This differentiation process is only possible due to the activation of a cascade of transcription factors like peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT enhancer binding protein (C/EBPs) family [1, 2, 10-12]. C/EBP family is constituted by five members: C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ and CHOP-10

[1]. Whereas some are associated with the adipogenesis process, like C/EBPα, C/EBPβ and C/EBPδ, others are related to the inhibition of this process, like CHOP-10 [11].

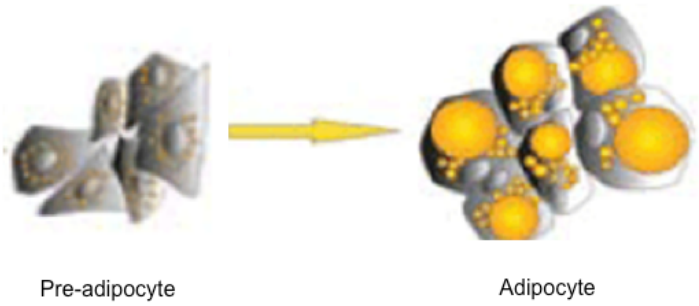

Figure 1.1: Pre-adipocyte differentiation into mature adipocytes.

Pre-adipocytes differentiation is induced with cAMP-elevating agents. When in the differentiated state, adipocytes are able to accumulate triglycerides inside lipid droplets.

The differentiation process has been extensively studied using cell lines, like 3T3-L1, and primary cultures, like mouse embryo fibroblast (MEF) [12]. This process can only be initiated when pre-adipocytes are post-confluent and growth arrested [1, 10-12]. When these two conditions are accomplished and the differentiation inducers are added to the *in vitro* culture, pre-adipocytes initiate the mitotic clonal expansion (MCE) with, at least, two rounds of mitosis [13].

Immediately before the initiation of the MCE, C/EBPβ is expressed (see figure 1.2). C/EBPβ plays a role in two different stages of the differentiation process: it initiates the mitotic clonal expansion and activates the expression of C/EBPα and PPARγ [11, 13, 14].

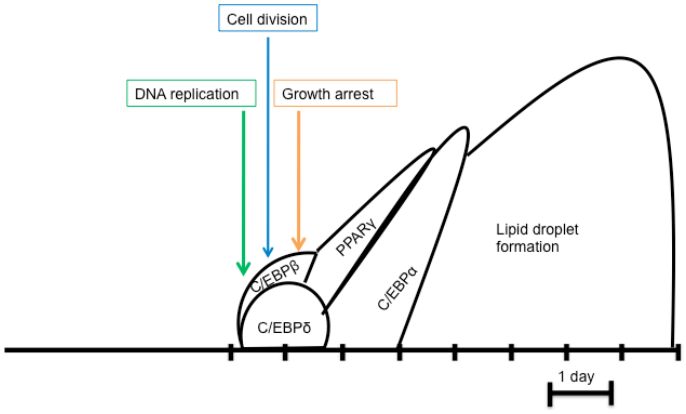

Figure 1.2: Progression of 3T3-L1 pre-adipocyte differentiation

The periods of gene expression during the differentiation programme have the gene name. C/EBP, CCAAT/enhancer binding protein; PPARγ, peroxisome proliferator activated receptor γ. Adapted from [15].

When cells enter the G1 - S phase of MCE, C/EBPβ gains DNA-binding affinity by being phosphorylated in Thr^{188} and Ser^{184} or Thr^{179} by mitogen activated protein kinase (MAPK) and GSK3β, respectively [14]. Then, PPARγ and C/EBPα expression is induced and cells enter in the terminal

differentiation phase (figure 1.2). The delayed expression of these two transcription factors is critical, since they both have anti-mitotic activity [12, 13]. Moreover, PPARγ and C/EBPα will function together as transcriptional activators of a large group of genes, like those involved in lipid metabolism that confer the adipocyte phenotype [10, 11, 16]. In addition, they regulate each other to maintain their levels during and after the differentiation process [17].

Although PPARγ and C/EBPα act together during differentiation, PPARγ has a dominant action. This was observed when PPARγ null embryonic stem cells did not differentiate into adipocytes, whereas in C/EBPα null stem cells, PPARγ was able to promote differentiation [1, 18]. PPARγ expression is not restricted to induction of adipogenesis, it is also needed to maintain the differentiated state, otherwise adipocytes lose their ability to accumulate lipids and express their adipocyte markers [1]. PPARγ is only expressed two days after the beginning of differentiation [15]. Its activation is correlated with the loss of DNA binding activity of E2F/DP, which is involved in cell division [19]. PPARγ is a *cis*-acting element [19] that promotes gene expression via formation of a heterodimeric DNA-binding complex with the retinoid receptor α (RXRα) [16]. To have adipogenic activity PPARγ needs more than being transcribed, it needs to be activated [15, 20]. Such activators can be micromolecular concentrations of long-chain fatty acids, like linoleic acid [19-21]; synthetic compounds, like thiazolidinediones (TZDs) [19, 20]; or naturally occurring eicosanoids, like prostaglandins [21]. These activators also differentially activate the other PPAR family members, for example, some eicosanoids, like 8(S)- hydroxyeicosatetraenoic acid, only activate PPARα [21].

PPARγ possesses two isoforms, PPARγ1 and PPARγ2, which are generated by alternative splicing and alternative promoter usage [1, 16]. These two isoforms are identical peptides, although PPARγ2 has 30 additional amino acids at the N-terminus [11]. These two isoforms are important for adipogenesis, although PPARγ2 plays an indispensable role [22]. When PPARγ1 and PPARγ2 expression was abolished in 3T3-L1, these cells were unable to differentiate [22]. However, with the addition of exogenous PPARγ2 the differentiation was restored, whereas when the exogenous PPARγ1 was used no differentiation occurred [22]. In another study using PPARγ2 knockout mice, the same conclusion was obtained [23]. These animals showed a decrease in the overall amount of AT, less lipid accumulation, reduced expression of the adipogenic genes and also insulin resistance [11, 23].

1.2.1 Adipogenic transduction pathways

Some of the pre-adipocyte differentiation inducers added to the *in vitro* cultures are glucocorticoids, like dexamethasone, or insulin [15]. Both of these inducers lead to the activation of cAMP-responsive element-binding protein (CREB) [24], see figure 1.3. This protein is activated by the phosphorylation of Ser^{133}. When phosphorylated, CREB activates the transcription of some genes like C/EBPβ that, at the right time, activates the transcription of other genes like PPARγ and C/EBPα [24]. CREBs activation can occur either through the increase in AMP cyclic (cAMP) levels that activate protein kinase A (PKA), which in turn activates CREB [25-27]; or through insulin pathway that activates extracellular signal-regulated kinase (ERK) 1/2 pathway, responsible for phosphorylating Ser^{133} of

CREB [24, 25]. Although PKA seems to have a crucial role during the beginning of the differentiation process, it is described that the continuous activation of PKA is responsible for inhibition of late differentiation. These results were obtained by the demonstration that 72 hours after the beginning of differentiation, cAMP levels and PKA activity are similar to basal [28]. It was also demonstrated that the continuous activation of PKA resulted in blockage of differentiation [28], whereas others demonstrated that PKA inhibitor (H-89) reduced the time span needed for full adipogenesis [29], once again, proving that PKA inhibits adipogenesis.

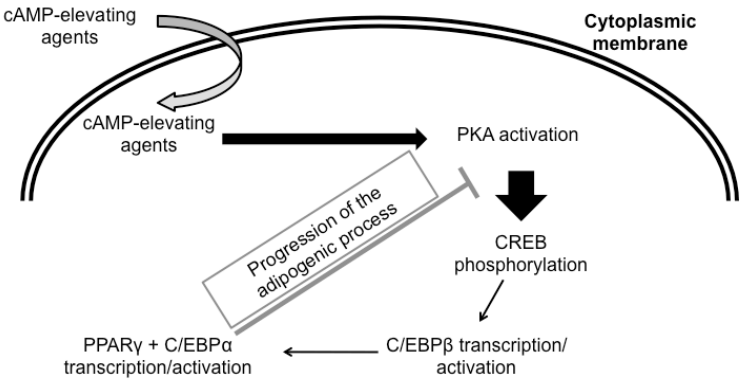

Figure 1.3: Adipogenic transduction pathways.

PKA activation by the increased cAMP levels leads to CREB phosphorylation. This transcription factor activates the transcription of C/EBPβ, which in turn activates PPARγ and C/EBPα transcription. For the successful progression of adipogenesis PKA needs to be inactivated. PKA, protein kinase A; CREB, cAMP-responsive element-binding protein; c/EBP: CCAAT/enhancer binding protein; PPARγ: peroxisome proliferator-activated receptor γ;

1.3 Metabolic function

WAT is the specialized tissue, of the human body, in accumulating energy in the form of triacylglycerols (see figure 1.4), also called neutral fats or triglycerides [30]. Each molecule of triacylglycerol is constituted by three molecules of esterified fatty acids (FA) and one molecule of glycerol [30].

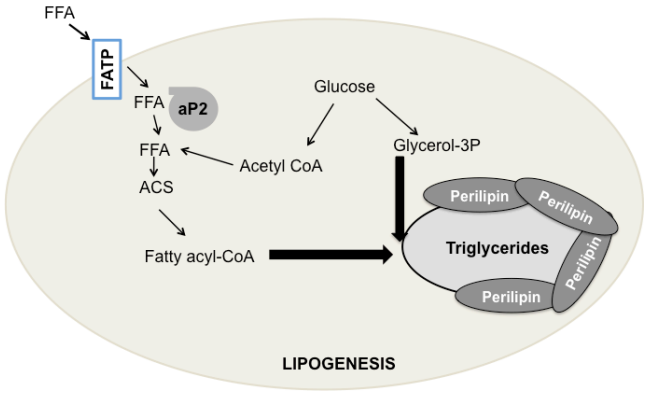

Figure 1.4: **Lipid metabolism in adipocytes**.

ACS, acyl-coenzyme A synthetase; aP2, adipocyte fatty acid binding protein; FATP, fatty acid transporter protein; glycerol 3-P, glycerol 3-phosphate; FFA, free fatty acids;. Adapted from [2].

1.3.1 Lipid droplets formation

In the differentiated state, adipocytes can either synthesize FA *de novo* or accumulate them from the dietary lipids [2]. To be accumulated from dietary lipids, triacylglycerides are transported through the blood plasma in lipoprotein transport particles, like chylomicrons or very low density lipoproteins (VLDL), to the adipose tissue [31]. In order to enter the cell, they are digested by lipoprotein lipase (LPL) and, as fatty acids, are able to enter the cell through fatty acid transporters [2, 31]. These transporters can be protein CD36 (human homologue to the murine fatty acid translocase, (FAT), fatty acid transport protein (FATP) and fatty acid binding protein (FABPpm) [31]. When in the cytoplasm, FA are carried out by cytoplasmatic binding proteins, like aP2, to acyl CoA synthase reaction site [31]. This enzyme catalyses the reaction of FA with an acetyl-CoA, originating fatty acyl-CoA [32]. Afterwards, this molecule reacts with glycerol-3-P, which is produced during glycolysis, originating the triglyceride molecule [32]. During the fatty acids *de novo* synthesis, the acyl-CoA molecule comes from an acetyl-CoA molecule that was produced from pyruvate in the glycolytic pathway, inside the mitochondria [30]. This acyl-CoA molecule, together with a glycerol-3-P molecule gives rise to a triglyceride molecule [30]. These triglyceride molecules (TAG) are kept inside lipid droplets protected by proteins called perilipin [33].

1.3.1.1 Perilipin

Perilipin belongs to a family called PAT family where are also included adipose differentiation-related protein (ADRP) and TIP-47 [34]. Perilipin is expressed in differentiated adipocytes and, under non-stimulated conditions, it protects lipid droplets from the action of lipases and therefore from lipolysis [35]. In pre-adipocytes or in differentiating fibroblasts the small lipid droplets are protected by ADRP that is replaced by perilipin when adipocytes become differentiated [33]. Perilipin has two

isoforms: perilipin A and perilipin B, that result from mRNA splicing of a single perilipin gene [33]. Perilipin A has six phosphorylation sites, whereas perilipin B only has three phosphorylation sites [34]. Perilipin A is found in the outer surface of the lipid droplet and is a critical component of a scaffold that stabilizes the lipid droplet [35]. This distribution at the surface of the lipid droplets is the main mechanism that prevents lipases from reaching TAG, thus inhibiting lipolysis [35].

1.3.2 Lipolysis

Lipolysis is the controlled process of hydrolysis of TAG, with consequent release of three fatty acid molecules and one glycerol [33, 36]. This process is triggered by hormonal stimulus, like catecholamines; by agents that elevate cAMP levels, like forskolin; or by cAMP analogous [33, 37]. When a catecholamine binds its β-adrenergic receptor, occurs the activation of a stimulatory G-protein (Gαs) that activates adenyl cyclase [33, 37]. Consequently, cAMP levels rise and activate the regulatory subunits of PKA [33, 37]. When in its active form, PKA phosphorylates the two key enzymes of the lipolysis process: perilipin and hormone sensitive lipase (HSL) (see figure 1.5) [33, 37]. As described above, perilipin plays a crucial role in preventing lipolysis [33, 35, 37]. However, it is also a crucial enzyme in "allowing" lipolysis [33, 35]. When this enzyme is not phosphorylated it is anchored to the lipid droplet and contributing to the barrier formation against the action of lipases like HSL. However, when it is phosphorylated by PKA, perilipin plays a different role [35]. When PKA phosphorylates these two proteins, perilipin changes its conformation allowing HSL to translocate to the surface of the lipid droplet [33]. While leaving the surface of the lipid droplet, perilipin facilitates the interaction between HSL and the lipid droplet [35]. HSL is a serine hydrolase regulated by reversible phosphorylation, whose metabolic function is to catalyse hormone-stimulated lipolysis [36]. This enzyme is present in the cytosol of adipocytes in its unphosphorylated form [33, 36]. Although HSL has several phosphorylation sites, the crucial for its activation as a lipase are Ser^{659} and Ser^{660} [33, 36]. It is important to note that the perilipin-HSL interaction is required, otherwise HSL alone is not able to reach the lipid droplet surface [33] and to initiate the hydrolysis of the ester bonds. Meanwhile, perilipin leaves the surface of the lipid droplet and its lysosomal degradation is started [38]. Because of this degradation it is possible to observe a decrease in perilipin levels with the concomitant increase of lipolysis [38].

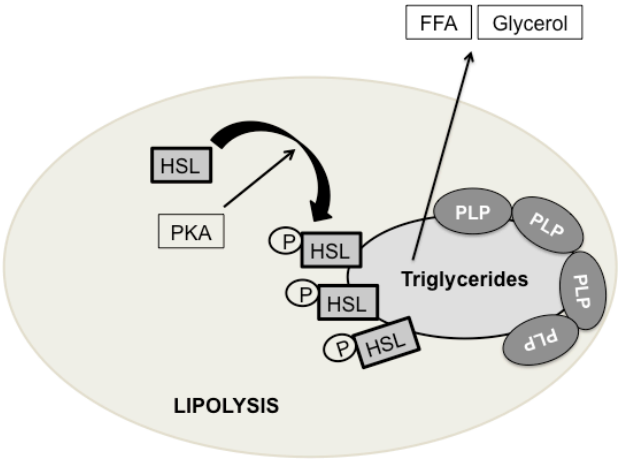

Figure 1.5: **Lipolysis transduction pathways in a human adipocyte.**

Lipolytic pathway is described. PKA, protein kinase A; HSL, hormone sensitive lipase; FFA, free fatty acid; P-phosphorylated; PLP, phosphorylated perilipin. Adapted from [37].

1.4 Endocrine function

Besides being a specialized organ in accumulating energy, the adipose tissue is also seen as an endocrine, autocrine and paracrine organ [9]. Such action is accomplished by the production of several molecules, also called adipocytokines or adipokines [39], whose actions influence not only the metabolic activity of the adipose tissue but also other tissues like the brain, muscles and liver [9, 40]. As outlined in table 1, adipocytokines have effects on multiple biological systems like energy homeostasis (lipid and carbohydrate metabolism, appetite, thermogenesis), immune system, reproductive function, hemostasia/coagulation, blood pressure and angiogenesis, see revision [41]. As referred before, the adipose tissue possesses other cell types besides adipocytes that can be the source of some of these adipocytokines [39].

Some of these molecules are leptin, adiponectin, visfatin, omentin, resistin, retinol-binding protein 4 (RBP 4), tumor necrosis factor – α (TNF-α), Interleukin-6 (IL-6) and other cytokines, see revision [42]. The former four molecules have anti-diabetic functions, while the later four molecules tend to raise blood glucose levels (see figure 1.6) [40, 43].

Leptin was the first adipokine to be discovered and is secreted almost exclusively by fat, more specifically by adipocytes [40]. Leptin has a wide spectrum of endocrine and paracrine functions that include appetite regulation, modulation of hepatic and adipose tissue-related insulin secretion and activity, modulation of steroid production in the ovaries and adrenal cortex, effects on reproductive physiology, and in hematopoietic and immune development, see revision [39]. Leptin acts through a leptin receptor that is highly expressed in the mediobasal hypothalamus, more specifically in the arcuate nucleus, ventromedial nucleus and dorsomedial nucleus, see revision [42]. When activated, leptin receptor represses orexigenic pathways, like those involving neuropeptide Y (NPY), and induces anorexigenic pathways, for example, those involving proopiomelanocortin (POMC) [40]. These actions include repressing of food intake, increasing energy expenditure and anti-hyperglycaemic actions that

result in improvement of insulin sensitivity in muscle and liver [40]. Animals and humans with mutations in lepin or in leptin receptor became obese, showing the importance of this adipokine in regulating food intake and energy expenditure [6]. Morrison et al., (2005) demonstrated that this effect of leptin mutations is due to increased NPY expression in the arcuate nucleus, which leads to increased food intake and decreased energy expenditure [44, 45]. In the same study, it was also observed that when leptin was injected in leptin deficient mice, NPY mRNA levels were decreased in the arcuate nucleus [44]. These results suggest that leptin action might also be through modulation of other peptides secretion [44].

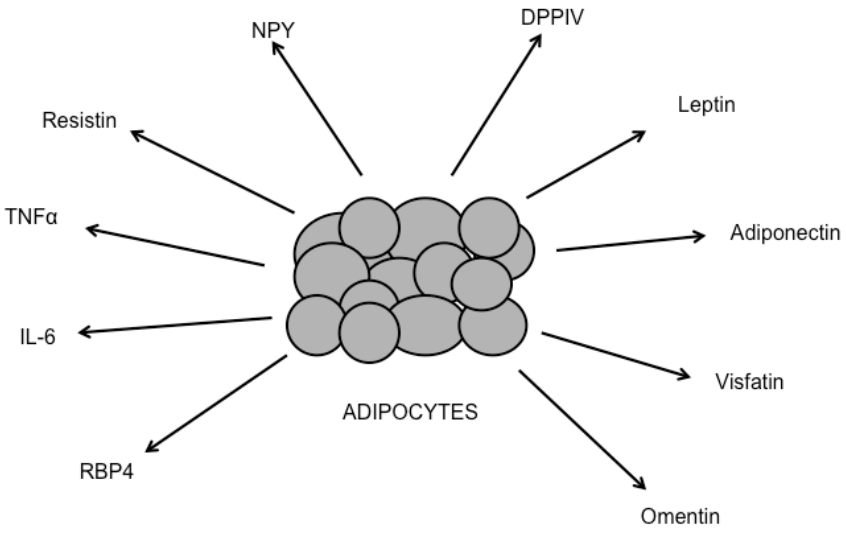

Figure 1.6: **Examples of molecules secreted by the adipose tissue with varied effect**

Leptin, adiponectin, visfatin and omentin are adipokines with anti-hyperglycaemic effects. Resistin, tumour necrosis factor-α (TNFα), interleukin 6 (IL6) and Retinol-binding protein 4 (RBP4) are molecules with pro-hyperglycaemic activity. Neuropeptide Y (NPY) and dipeptidyl-peptidase IV (DPPIV) with adipogenic activity. Adapted from [40].

Adiponectin is a 30 kDa protein also known as apM1, GBP28, AdipoQ and ACRP30 [40]. It has an amino-terminal collagen-like domain and a carboxyl-terminal globular domain that mediates multimerization [40]. Adiponectin is specifically expressed in differentiated adipocytes and suffers posttranslational modification [46]. This adipokine circulates as a trimer, hexamer or as a higher-order multimer that circulates in plasma in such high concentrations that account for 0.01% of all plasma protein [40]. Adiponectin has two different receptors, AdipoR1 and AdipoR2, being the former primarily expressed in muscles, and the latter primarily expressed in the liver [41]. Adiponectin effects depend on circulating levels, on the properties of the different adiponectin isoforms and also on tissue specific expression of adiponectin receptors [46]. Nevertheless, there are some general functions associated with adiponectin, like anti-diabetic and anti-inflammatory functions [47]. The anti-diabetic functions are related with the increased levels of adiponectin when TZDs are administered, resulting in improved insulin sensitivity [40].

Visfatin is produced by visceral adipose tissue and was firstly identified in immune cells as a pre-B-cell colony-enhanced factor (PBEF) [40]. It can directly bind and activate the insulin receptor

which results in an enhanced glucose uptake [43]. Because its circulating levels are less than 10% compared to insulin levels, these two molecules do not compete for receptor binding [40]. A recent article suggested that visfatin not only activates the insulin receptor, but also regulates insulin secretion and several β-cell function-associated genes in mouse [48].

Omentin is secreted by stromal-vascular cells present in visceral fat [32]. It acts as an insulin sensitizer rather than insulin mimetic, resulting on positive effects in glucose uptake [32]. In humans and macaques it is produced in high quantities, but in mice no production was detected [40]. Although omentin levels decrease with obesity and insulin resistance, the mechanism of action is still unknown [43].

Retinol-binding protein 4 (RBP4) is a member of the lipocalin superfamily and its expression is regulated by changes in the glucose transporter type 4 (GLUT4) [40]. High serum levels are associated with insulin resistance in humans with obesity, type 2 diabetes *mellitus* and in lean non-diabetic people with family history of type 2 diabetes *mellitus*. RBP4 overexpression in mice, also resulted in impaired insulin action both in muscle and liver [32].

The adipose tissue also produces some cytokines, like **interleukin 6 (IL-6)**, and **tumour-necrosis factor α (TNFα)** [40]. Although TNFα is secreted in the adipose tissue, it is not secreted by adipocytes, but by macrophages that surround adipose tissue [46]. This adipokine also plays a role in glucose homeostasis, by decreasing insulin sensitivity [39]. Its levels are elevated in obese conditions and in other insulin-resistant cases [39]. Moreover, when TNFα expression was blocked, insulin sensitivity was restored, both *in vitro* and *in vivo* [40]. On the other hand, IL-6 is produced by adipocytes and circulates in multiple glycosylated forms [46]. Its expression and circulating levels are positively correlated with obesity, glucose intolerance and insulin resistance [39]. When peripherally administrated, IL-6 reduces the expression of insulin receptor signalling components, decreases adiponectin secretion and inhibits adipogenesis [46].

Resistin, also known as FIZZ3, is a small inflammatory molecule with hyperglycaemic action [40]. Although some authors indicate that it is secreted by adipocytes [49], it is still controversial since new data suggests that resistin is secreted by macrophages [50] or other stromal cells present in the adipose tissue [51]. Resistin circulates in the plasma in several multimeric forms, but are those with small weight that seem to have an effect at the cellular level [6]. Resistin reduces glucose uptake in muscles and is repressed by TZDs [6]. This molecule can also modulate the secretion of other molecules, such as NPY [52]. This was observed when resistin was centrally administered to mice, resulting in increased NPY production in the arcuate nucleus [52]. Resistin effects on glucose production were blocked in mice lacking NPY [52].

In conclusion, the adipose tissue has very important actions in regulating body homeostasis. Not only by accumulating energy but also by signalling to the brain when food intake is needed and when is not, through the production of peptides, like NPY [53]. Adipokines also support the other tissues with all the energy that they need to work properly [32].

Table 1: Examples of some factors secreted in the adipose tissue and respective function [6, 32].

Adipokine	Biological effect
Leptin	Signals to the CNS about the body's energy stocks
Adiponectin	Increases sensitivity to insulin; antiinflammatory; attenuates the progression of atherosclerosis
Visfatin	Insulin-mimetic; predominantly produced by visceral fat
Omentin	Enhances insulin-stimulated signals and glucose uptake but it is not insulin-mimetic
TNFα	Lipolytic; increases energy consumption; reduces sensitivity to insulin
IL-6	Proinflammatory, lipolytic, reduces sensitivity to insulin
Resistin	Increases insulin resistance
RBP4	Reduces insulin sensitivity; Impaires insulin action in the muscle

1.5 Dipeptidyl peptidase IV

1.5.1 Structure and localization

Dipeptidyl peptidase IV (E.C. 3.4.14.5) is a 110 kDa glycoprotein, also known as DPPIV or CD26 [5]. This enzyme is a ubiquitous and multifunctional molecule that exists in both soluble and membrane bound form. DPPIV is also a homodimer but it functions only while in its dimer form [5, 54-57]. In its membrane form, DPPIV has three regions: a cytoplasmatic domain with 6-amino acids, a hydrophobic transmembranar region with 22-amino acids and an extracellular domain with 738-amino acids [56]. When the hydrophobic N-terminal domain is cleaved [58] by chymotrypsin-like or pepsin-like enzymes [59], DPPIV is released into the plasma, becoming soluble, though maintaining both substrate specificity and susceptibility to inhibitors [57]. While in its soluble form, DPPIV can be found not only in the blood plasma, but also in the cerebrospinal fluid and semen [60, 61]. As a membrane protein, it is widely expressed in various tissues and organs, including the exocrine pancreas [62], kidneys [63], gastrointestinal tract [64], thymus [65], lymph nodes [66], uterus [67], placenta, prostate [68], adrenal [69], sweat [70], salivary and mammary glands [70], endothelia of spleen [71], lungs [63], brain [69], and vessels supplying the liver [61, 72, 73]. In addition, this enzyme is also present in lymphocytes, as the cell-surface CD26 T-cell-activating antigen and also as a membrane antigen of both Natural Killer (NK) and B cells [61, 73-75].

1.5.2 Function and substrates

DPPIV is a multifunctional protein involved in several processes like cell growth [76] and differentiation [77], adhesion [60], immunomodulation [78], metabolism [79], endocrinology, cancer biology and apoptosis [68, 80]. In order to accomplish these processes, DPPIV has three mechanisms of action

[78]: (i) cell-extracellular matrix interactions [60] (ii) co-stimulatory role in the immune system [5, 60, 78, 81, 82] and (iii) cleavage of biologically active molecules [60].

The extracellular matrix interactions are characterized by the ability of this enzyme to bind some ligands, like adenosine deaminase (ADA), kidney Na^+/H^+ ion exchanger 3 [60, 78]. DPPIV is also able to bind fibronectin and collagen and the association of DPPIV with fibronectin, in lung cancer cells, is responsible for growth arrest of these cells [83]. In cancer cells DPPIV has anti-proliferative and anti-oncogenic effects and is down-regulated [68, 78]. Although it is not known the exact mechanism of action, DPPIV down-regulation will create a proteolytic imbalance of the extracellular regulatory proteins within the tumour environment [68, 69, 84] which is characteristic of malignancy [78].

DPPIV also has a very important role in the immune system. This enzyme is not only able to regulate multiple T-cell functions, like maturation, activation, migration and interaction with antigen-presenting cells; but is also able to regulate proliferation and activation of B-cells and NK cells [60, 78]. Furthermore, DPPIV can also cleave several immunoregulating cytokines [78]. When in the surface of T-cells, the signal transduced by CD26 co-stimulates the T-cell receptor CD3 pathways, leading to T-cell activation [85]. CD26 activation occurs through caveolin-1 or CD45 and leads to tyrosine phosphorylation which increases phosphorylation of several molecules like $p56^{lkc}$, $p59^{fyn}$, zeta associated protein-tyrosine kinase of 70000MW (ZAP-70) and MAPK [5, 60, 78, 86]. Studies also showed that in some autoimmune diseases, like Multiple Sclerosis and Arthritis, the CD26 expression is up-regulated in T-cells, B-cells and NK-cells [5, 78]. Taking all together, it can be concluded that DPPIV inhibitors are useful tools for immune suppression in autoimmune diseases [5, 78, 82].

The DPPIV ability to cleave molecules is restricted to those enzymes having a Xaa-Pro or Xaa-Ala dipeptides, from the N-terminus of polypeptides (where Xaa is any amino acid except Pro) [5, 54, 61, 74]. These active molecules, as outlined in table 2, include a variety of neuropeptides, chemokines and circulating hormones, among others [54, 56, 61]. When cleaved by DPPIV, these molecules can either be inactivated, like gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP1); their receptor specificity altered, as NPY and peptide YY (PYY); or their activity reduced, like monokine induced by gamma interferon (MIG) [61, 78, 87].

Table 2: DPPIV substrates [61, 78, 87, 88]

Neuropeptides	Neuropeptide Y Endomorphin Substance P Beta-casomorphin Pituitary adenylate-cyclase-activating polypeptide (PACAP) Vasoactive intestinal peptide (VIP)
Other regulatory peptides	Gastric inhibitory peptide (GIP) Gastrin-releasing peptide (GRP) Glucagon-like peptide 1 (GLP-1) Glucagon-like peptide 2 Growth-hormone releasing factor (GHRF) Peptide YY (1-26)
Chemokines	Eotaxin Monokine induced by gamma Interferon (MIG) Interferon-inducible protein-10 Chemokine lingand 5 (RANTES) Macrophage-derived chemokine Macrophage inflammatory protein-1beta Monocyte chemotatic proteins 1-3

Some of these substrates have different functions, depending on the tissue where they are produced and on the presence of their receptors [87]. The adipose tissue, has receptors for GLP-1 [89], GIP [90], Substance P (SP) [91-93], Pituitary adenylate-cyclase-activating polypeptide (PACAP) and NPY [94].

1.5.2.1 DPPIV substrates and their functions in the adipose tissue

GLP-1 is a peptide released by the enteroendocrine cells (L-cells), in the gut [95, 96]. Besides stimulating the adipose tissue to produce leptin, this peptide also decreases fat storage [89, 97]. GLP-1 enhances insulin-stimulated glucose metabolism in 3T3-L1 adipocytes: one of several potential extrapancreatic sites of GLP-1 action [98]. This process is not accomplished through lipolysis, but via direct modulation of adipocyte metabolism [97]. Nevertheless, this ability to modulate the adipocyte metabolism is not present in obese condition, suggesting an obesity-induced adipocyte resistance to GLP-1 [97].

GIP is synthesized and secreted from K cells in the intestinal epithelium [99]. It has been shown that this peptide has a direct effect on adipocytes by dose dependently stimulating lipoprotein lipase activity and also fatty acid synthesis [90]. In addition, mice lacking GIP receptor (GIPr (-/-)) fed with a high fat diet were clearly protected from both obesity and insulin resistance [100].

SP is released from the enteric nerves, sensory neurons and also from inflammatory cells of the lamina propria during intestinal inflammation [91]. This peptide also plays a role in the adipose tissue by increasing pre-adipocyte viability and proliferation, and also by decreasing apoptosis [91].

PACAP is released upon stimulation of the parasympathetic nerves [101]. By alternative splicing this peptide can be released in two forms, PACAP38 and PACAP27, having the former more affinity to DPPIV [87]. PACAP function in the adipose tissue depends on the presence of insulin, i.e., if insulin is present, this peptide potentiates glucose uptake which leads to lipogenesis; if insulin is absent, PACAP becomes lipolytic [94].

NPY is synthesized both in the hypothalamus and in the adipose tissue [102, 103]. In the adipose tissue, NPY increases expression and activity of lipoprotein lipase, which leads to increased lipogenesis [102]. It also has a very strong anti-lipolytic effect, resulting in increased weight gain [102, 104]. The effect of NPY in the adipose tissue will be discussed later on section 1.6.3.

1.6 Neuropeptide Y

NPY is a 36 amino acid peptide amidated in the C-terminal [105]. It belongs to the same family as peptide YY (PYY) and pancreatic polypeptide (PP), with whom shares 70 and 50% of sequence identity, respectively [105]. This peptide was first discovered in the porcine brain [53] and is the most abundant peptide in the human brain and very well conserved among species [106]. The NPY gene is located in the human chromosome 7 and is expressed mainly in the hypothalamus, more specifically, in the paraventricular nucleus, arcuate nucleus, suprachiasmatic nucleus, median eminence and dorsomedial nucleus [53]. In the sympathetic neurons, NPY is colocalized with norepinephrine [106]. Besides neuron cells, there are also other cells able to secrete NPY like, for example, liver, heart, spleen, endothelial cells of blood vessels and adipose tissue [53, 103].

NPY is a neurotransmitter involved in the regulation of several actions like, control of food intake, regulating the activity of neuroendocrine axes under poor metabolic conditions, vasoconstrictive actions, cardiovascular regulation, blood pressure, catecholamine secretion, energy homeostasis, neuroendrocrine regulation, memory processing, anxiety, body temperature, lipogenesis, among others [53, 105-107].

1.6.1 NPY synthesis

When synthesized, NPY is a precursor peptide of 97 amino acids: preproNPY that, after cleavage, loses its signal sequence, see figure 1.7 [53]. This new peptide, with 69 amino acids, is further converted into NPY_{1-39} and C-flanking peptide of NPY (CPON) (30 amino acids) by a group of enzymes called pro-converting enzymes [106]. Afterwards, a carboxypeptidase-like enzyme cleaves two more amino acids giving rise to NPY_{1-37}, which is amidated in the C-terminal, by a peptidyl-glycine-α-amidating monooxygenase, losing one amino acid and becoming NPY_{1-36} [106]. Although this peptide is

already in its biologically active form it can be further be cleaved by aminopeptidase P or DPPIV, like described above [106].

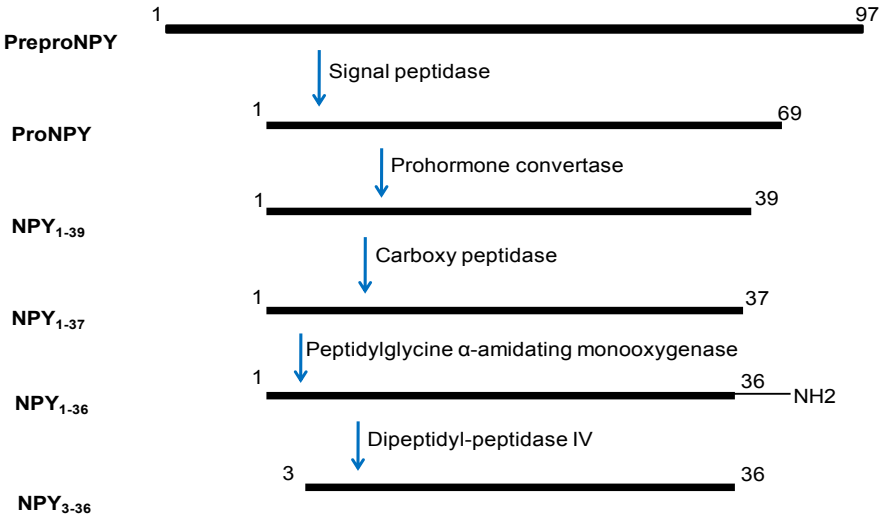

Figure 1.7: **Synthesis and post-transactional modifications of neuropeptide Y.**
Adapted from [106].

1.6.2 NPY receptors

NPY acts through six G-protein-coupled receptors: Y_1, Y_2, Y_3, Y_4, Y_5 and y6 [106]. This type of receptor is associated with a seven-transmembrane-helix (7TM) receptors (see figure 1.8) that, when activated, suffers a conformational change which in turn activates a G protein [30]. NPY receptors are Gi/Go coupled receptors that when activated inhibit the action of adenyl cyclase [108]. This action results in decreased cAMP levels that in turn prevent PKA activation [30]. Besides decreasing cAMP levels, NPY receptors are also able to increase intracellular calcium levels [108, 109].

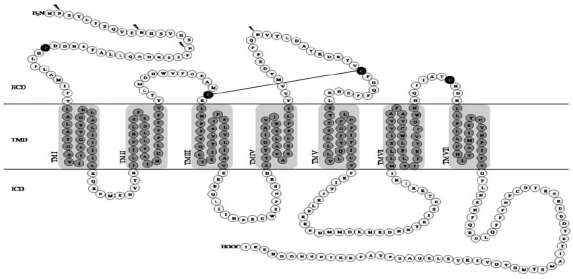

Figure 1.8: **Representation of the amino acid sequence of the G-protein coupled Y_1 receptor** [110].

1.6.2.1 NPY Y_1 receptor

NPY Y_1 receptor gene is localized in the chromosome 4q(31,3-32) and it is expressed in the brain, heart, kidneys and gastrointestinal tract, see revision [106]. NPY binding activity to this receptor is largely impaired when enzymes, like DPPIV, cleave the NPY N-terminal peptides [53]. However, when the C-terminal peptides are modified, NPY retains its full binding capacity to Y_1 receptor, suggesting that this neurotransmitter binds this receptor through its N-terminal region [53]. The main effects of NPY, mediated by Y_1 are vasoconstriction [53], increased appetite [111], decreased depression [112] and anxiety [106], activation of the neuroendocrine axes [106], and proliferation of smooth muscle cells [113], progenitor cells of the hippocampus [114], pancreatic β cells [115], Muller cells [116] and tumour cells [117].

1.6.2.2 NPY Y_2 receptor

The NPY Y_2 receptor is located in the chromosome 4q31, close to the NPY Y_1 and Y_5 receptor locus [106]. Its expression is found in the central and peripheral nervous system, gut, certain blood vessels and adipose tissue, see revision [53]. This receptor does not require the NPY N-terminal sequence, since it binds NPY_{3-36} and NPY_{1-36} with the same affinity [53]. NPY Y_2 receptor also plays a role in control of appetite [118] and angiogenesis [113], neurotransmitter release [106].

1.6.2.3 NPY Y_3 receptor

NPY Y_3 receptor is called the NPY preferring receptor since it shows 10-fold higher affinity for NPY than for PYY [53]. Although it has never been cloned nor well characterized [106], it can be found in several peripheral tissues, like rat superior cervical ganglia sympathetic neurons [119], rat cardiac ventricular membranes [106] and rat distal colon [120]. In human adrenal medulla mediates NPY-induced secretion of catecholamines [121].

1.6.2.4 NPY Y_4 receptor

NPY Y_4 receptor is located in the chromosome 10q 11-12 [106] and was first identified as a PP receptor [53], because it binds PP with higher affinity than NPY or PYY [122]. This receptor is mainly expressed in the colon, small intestine, prostate [106], pancreas, smooth muscle cells and in various regions of the brain, like hypothalamus [123-125]. Its affinity to NPY is only moderate, however PP is the primary endogenous ligand. Therefore, PP effects like inhibition of exocrine pancreatic secretion or induction of gall bladder relaxation, might occur through this receptor [53].

1.6.2.5 NPY Y_5 receptor

NPY Y_5 receptor gene is located in chromosome 4q32, in the same locus as NPY Y_1 receptor, although their transcription is in opposite directions [106]. NPY Y_5 receptor is activated by NPY, PYY, PYY analogs and fragments of peptides, such as NPY_{3-36} and PYY_{3-36} [126]. It is mainly expressed in the hypothalamus, where it stimulates the appetite [127]; and, at the peripheral level, is present in the intestine, ovary, testis, prostate, spleen, pancreas, kidney, skeletal muscle, liver, placenta and heart, see revision [53].

1.6.2.6 NPY y_6 receptor

NPY y_6 receptor is localized in the chromosome 5q31 [106] and, like NPY Y_4 receptor, shows higher affinity to PP than to NPY or PYY [128, 129]. This receptor is present in mice, rabbits, primates and humans, although its physiological action is not yet known [130-132]. y_6 receptor mRNA was also detected in several tissues like hypothalamus, hippocampus, gut and adrenal glands of rabbits; and also in skeletal muscle and hypothalamus of humans [130, 131, 133].

1.6.3 NPY and NPY receptors in the adipose tissue

The presence of NPY and its receptors in several tissues and the important actions played by this neuropeptide, raised the question of whether NPY would also play a role in the adipose tissue. When synthesized in the hypothalamus, NPY has a very potent orexigenic action [134]. When centrally administered, NPY causes a strong increase on food intake (hyperphagia) and a decrease on energy expenditure, resulting in weight gain [135]. Recent studies showed that both NPY and some of its receptors are synthesized in the human [135], pig [136], mouse [94] and rat [135] adipose tissue as well as in a murine pre-adipocyte cell line (3T3-L1) [94], suggesting that this peptide might play a role directly in the adipose cells.

1.6.3.1 NPY role on lipid accumulation

NPY expression levels are not always constant and there are many factors influencing NPY expression levels [106]. Nevertheless, it is also important to understand in which situations NPY expression levels are upregulated in the adipose tissue. During the early life of a programmed rat model of increased adiposity, scientists observed that NPY levels were 6-fold higher [103]. In the same study using as animal model the obese Zucker rats, NPY mRNA expression levels were also increased 2-fold [103]. And, because insulin stimulates NPY secretion, in cases of hyperinsulinemia, NPY levels should also be elevated [102]. Furthermore, it was also described that NPY has a direct

effect on adipocytes by promoting cell proliferation, lipid accumulation and cell differentiation in a 3T3-L1 cell line [94, 103]. Moreover, when NPY was subcutaneously applied to mice and monkeys, it stimulated growth of the adipose tissue [137]. Although it is still not established if these effects in the adipose tissue are mediated by Y_1 or Y_2 receptor [103, 137], it is already accepted that NPY up-regulates both lipoprotein lipase and fatty acid synthase expression and activity, two key enzymes in lipogenesis [94, 102].

Apart from playing a role in lipogenesis, NPY also has a role in lipolysis [94, 104]. Like it was described above, for lipolysis to occur, cAMP levels must be elevated [34]. However, when NPY receptors are activated, cAMP levels decrease [108]. In fact, this was observed in primary human adipocytes where NPY inhibited lipolysis [94]. This effect was confirmed by adding a NPY antagonist (S.A.0204), resulting in increased lipolysis and consequently the total lipid content decreased [107]. Other study also showed that NPY injection in rats or mice, not only stimulated food intake, but also inhibited lipolysis [53]. In addition, it was also shown that under hyperinsulinemic conditions, NPY increased adipocytes size [102]. The NPY receptor that mediates this action is still controversial. Some groups indicate that NPY's anti-lipolytic effect is through Y_1 receptor [135, 138], proven by binding studies and using antagonists [102, 106, 135, 138, 139]; and others showed evidences of the activation of Y_2 receptors [94]. Nevertheless, it seems that in rat adipose tissue there is a dual control through Y_1 and Y_2 [135]. Y_5 involvement was also proposed since the addition of Y_5 receptor agonists stimulated the accumulation of triglycerides by inhibiting lipolysis [140, 141].

1.7 Dipeptidyl peptidase IV inhibitors

Dipeptidyl peptidase IV (DPPIV) inhibitors are a class of drugs mainly used in patients with Type 2 Diabetes *Mellitus* (T2DM) [61]. This disease is characterized by high circulating glucose levels that resulted from insulin resistance and further impaired insulin secretion by the β-cells in pancreas [142, 143].

The main target of DPPIV inhibitors is to increase the half life of some insulin-stimulating hormones, like, GLP-1 and GIP [74]. Inhibition of plasma DPPIV leads to enhanced endogenous GLP-1 and GIP activity, which ultimately results in the potentiation of insulin secretion by pancreatic β -cells and subsequent lowering of blood glucose levels, HbA1c, glucagon secretion and liver glucose production [61, 81, 142]. In addition, GLP-1 and GIP have beneficial effects on pancreatic β-cells, including increased β-cell survival and expansion of β-cell mass [61].

These inhibitors are associated with low risk of hypoglycaemia and weight loss or weight neutrality [144]. DPPIV knockout mice, besides being healthy, have resistance to hepatic lipid accumulation when fed with a high fat diet [61]. In addition, investigators suggested that inhibiting DPPIV the circulating free fatty acids were prevented from rising not due to inhibition of FA mobilization but because FA oxidation was increased [144-146]. Moreover, it was also observed that, by inhibiting DPPIV, both apolipoprotein and chylomicron lipid levels were decreased, suggesting that these inhibitors also might inhibit intestinal fat extraction [144, 146].

Several inhibitors can be already found in the market and some are still waiting for Food and Drug Administration (FDA) approval (see table 3).

Because until 2010 Vildagliptin, Sitagliptin and Saxaglipin were the only three gliptins commercialized in Europe and, except from vildagliptin, also approved by FDA, our work will focus on them.

1.7.1 Vildagliptin

Vildagliptin, also known as LAF237 or Galvus®, is commercialized by Novartis and is a selective inhibitor of DPPIV [147]. This drug has a half maximal inhibitory concentration (IC_{50}) of 3,5 nM [61, 148] and specificity (Ki) of 17 nM [148]. It is normally taken by T2DM patients at a dose of 100 mg, being rapidly absorbed with 85 % of bioavailability [148]. When in the plasma, its half-life is 90 minutes [148] whereas when in the enzyme-inhibitor complex is 135 minutes [143]. 15-30 minutes after oral administration, DPPIV is inhibited by almost 100 % and maintains more than 80 % of inhibition for 16 hours [147]. This enzyme-inhibitor complex is covalently bound and is reversible [61, 148, 149]. In addition, vildagliptin behaves as a slow-binding inhibitor with slow decline in potency with time, which suggests that it acts as a tight-binding inhibitor [143]. Nevertheless, this inhibitor has some adverse effects like increased risk of infection (nasopharyngitis and urinary tract infection), dizziness and nausea [150].

1.7.2 Sitagliptin

Sitagliptin (MK-0431, Januvia®) is produced by Merck and, like vildagliptin, is a selective inhibitor for DPPIV [147] with an IC_{50} of 18 nM and a Ki of 9 nM [61, 148]. Although its plasma half-life is 2-fold higher than vildagliptin [61], the enzyme-inhibitor complex only lasts for 80 minutes [143]. Sitagliptin's selectivity is also higher than vildagliptin's, however no differences on side effects were found, suggesting that this difference is not of great importance [61]. The recommended dose is 100 mg once daily [150] and DPPIV plasma activity was also inhibited almost 100 % after 15-30 minutes of oral administration and lasted superior to 80 % for more than 16 hours [147]. It has renal excretion, where approximately 80 % of the oral dose is excreted unchanged in the urine [150]. Sitagliptin has a different kind of action, since it is a non-covalent reversible inhibitor [61, 148, 149]. Nevertheless, this gliptin is also a slow binding inhibitor with slow decline in potency with time [143].

When administered to patients with T2DM it showed effective glycaemic control, both in fasting and post-prandial conditions [148]. In addition, sitagliptin decreased adipocyte size and enhanced post-prandial lipid mobilization and oxidation [151]. The adverse effects observed were elevated risk of infection (nasopharigitis and urinary tract), headache and upper respiratory tract infection [150].

1.7.3 Saxagliptin

Saxagliptin (BMS-477118, Onglyza®) is produced by Bristol-Myers Squib/Astra Zeneca/Otsuka Pharma and has an IC50 of 3.37 nM, a Ki of 0.6 nM [148] and the enzyme-inhibitor complex has a half-life of 713 minutes [143]. This inhibitor is also highly selective for DPPIV and the daily dose taken by patients with T2DM is 5 mg [150]. Saxagliptin is well absorbed, has a low plasma protein binding and is metabolized *in vivo* to form an active metabolite that is 2-fold less potent than the parent molecule [152]. Saxagliptin is also covalently bound to DPPIV in a reversible way [148, 152]. Like vildagliptin and sitagliptin, saxagliptin also behaves as a slow-binding inhibitor with a slow decline in potency with time [143]. This gliptin has a dissociation constant 5-8 fold slower than the dissociation constants of the other inhibitors [143]. Likewise, saxagliptin showed the longer duration of action and the greatest potency of all DPPIV inhibitors [143].

When administrated to T2DM patients, it significantly improved glycaemic control by decreasing fasting plasma glucose and post-prandial glucose, when added with sulphonylureas or TZDs [148]. This inhibitor also has adverse effects like arthralgia, cough, headache, nasopharingitis, nausea, upper respiratory tract infection and urinary tract infection [150].

One of the disadvantages pointed to this type of inhibitors, is its specificity among DPPIV family members. However the IC_{50} or K_i needed to inhibit these other members are much higher than those used in therapy [148].

Table 3: Examples of DPP IV inhibitors [5, 61, 73, 74, 81, 142, 149, 150, 153-156]

Name	Type of Action	Status
Vildagliptin (LAF-237)	Covalently bound, Reversible inhibitor	Approved in Europe; Approved in Portugal
Sitagliptin (MK-0431)	Non-Covalently bound, Reversible inhibitor	Approved by FDA in 2006; Approved in Portugal
Saxagliptin (BMS-477118)	Covalently bound,Reversible inhibitor	Approved by FDA in 2009; Approved in Portugal
Linagliptin (BI-1356)	Reversible inhibitor	Approved by FDA in 2011
Denagliptin (GSK823093C)	-	Discontinued
Alogliptin (SYR-322)	Reversible inhibitor	Rejected by FDA in 2009
Dutogliptin (PHX-1149)	Reversible inhibitor	Phase II trials
Carmegliptin (R-1579)	Reversible inhibitor	Phase II trials completed
Melogliptin (GRC8200)	-	-
Isoleucine thiazolidide (P32/98)	Non-Covalently bound, Reversible inhibitor	Phase II trials
NVP-DPP728	Covalently bound, Reversible inhibitor	Discontinued
PSN-9301	Reversible inhibitor	Phase II trials
NN-7201	Reversible inhibitor	Phase I trials
ALS 2-0426	-	Phase I trials
Aminomethylpyridine (R1438)	Reversible inhibitor	Phase III trials
ABT-279	-	Phase II trials

1.8 DPPIV and gliptins in the adipose tissue

Inhibition of DPPIV became one of the most promising treatments for T2DM mainly because it lowers glucose levels by regulating GLP-1 and GIP [157]. Later it was discovered that not only this enzyme is also secreted in the adipose tissue [158], but it might also play a role in the modulation of this tissue [74, 158]. T2DM patients treated with sitagliptin have a decrease in the hepatic input of non-esterified fatty acids that was correlated with lower fat depots and a decrease in adipocytes size [151]. These effects were due to an increase in post-prandial lipid mobilization and oxidation [146, 151]. During clinical trials with vildagliptin, sitagliptin and saxagliptin, no significant changes in body weight were observed [144]. However, vildagliptin treatment also showed an increase in post-prandial lactate and glycerol in the adipose tissue, together with a decrease in pyruvate and lactate in skeletal muscle [144]. Taking it together, vildagliptin seems to be promoting lipolysis in the adipose tissue and, at the same time, increasing fatty acid oxidation in skeletal muscle [144].

However, in another study, C57BL/6 mice fed with a high fat diet became obese, but when treated with sitagliptin for 12 weeks it was observed a decrease in body weight which was originated by a decrease in the adipose tissue and a decrease in inflammation in the adipose tissue [159]. The decrease in the amount of adipose tissue was due to a decrease in the number of large adipocytes resulting in increased number of small adipocytes [159]. In addition, $GcK^{+}/_{-}$ mice also fed with high fat diet, became obese with adipocytes hyperthrophy and, when des-fluo-sitagliptin was administred, the hyperthrophy was reduced [160]. Recently, another study was made where C57BL/6 mice were fed with high fat diet for 10 weeks to become obese and, after this period sitagliptin was given [161]. The results showed that this drug prevented the hyperinsulinemia, hyperglycaemia and dyslipidemia found in the non-treated obese animals [161]. Furthermore, WT mice showed accelerated weight gain and hyperinsulinemia, in contrast with DPPIV knockout mice that were resistant to the development of obesity and hyperinsulinemia [162]. This resistance was associated with reduced food intake and increased energy expenditure [162]. Moreover, Fischer 344 mutant (CD26 knockout mice) mice were also fed with high fat diet showing similar results: resistance to obesity and decreased food intake [163]. In addition, these animals also showed decreased blood glucose, increased insulin sensitivity and increased plasma levels of GLP-1 [163]. Other study with DPPIV deficient mice also showed similar results. These animals were fed with high fat diets, and showed reduced weight gain, when compared with wild type mice [164]. These differences were attributed to the reduction of intraabdominal fat depots. However, some contrasting results appeared in this study, since DPPIV knockout mice also showed increased NPY levels, which would lead to increased food intake [164]. Nevertheless, these animals still lost weight, suggesting that DPPIV action is directly on intraabdominal fat [164].

Finally, it was also necessary to understand if there was any difference in DPPIV expression and activity between lean and obese subjects. In obese people, DPPIV expression is 5-fold increased in visceral adipose tissue than in subcutaneous adipose tissue, contrasting with lean subjects where no difference was observed between fat depots [165]. DPPIV plasma concentration also appears to be positively correlated with adipocyte size, i.e., higher DPPIV concentrations correlate with bigger adipocytes [165]. Others showed that rats with a high fat diet, not only became obese, but also had

higher levels of plasma DPPIV, when compared with normal diet-fed rats [158]. These authors suggest that the post-prandial glucose elevation, which leads to visceral fat accumulation, may be responsible for the increase in plasma DPPIV [158]. The higher the levels of DPPIV, the lowest the levels of GLP-1 and GIP, resulting in impaired glucose tolerance [158].

The alterations in DPPIV expression have been associated with changes in methylation in the promoter region of DPPIV gene. In human melanoma cell lines [166] and T-cell leukemia [167], where DPPIV expression is repressed, the promoter region appears to be hypermethylated. In addition, women with higher DPPIV gene methylation also showed lower DPPIV expression in their visceral adipose tissue, resulting in better plasma lipid profiles [168].

1.9 Objectives of the present study:

DPPIV is a peptidase released by the adipose tissue whose expression is increased in obese conditions [158, 165]. Previous studies demonstrated that this enzyme stimulates adipocyte differentiation and lipid accumulation (Ana P Marques; Joana Rosmaninho-Salgado, unpublished data). These evidences suggest that DPPIV is involved in the adipocyte metabolism and that this involvement might lead to an increase in the amount of adipose tissue as well as increased adipocyte diameter. A new class of DPPIV selective inhibitors is being commercialized for the treatment of T2DM [61]. These inhibitors are called gliptins and are associated with low risk of hypoglycaemia and weight loss or neutrality [144]. Three types of gliptins are already in the market: vildagliptin, sitagliptin and saxagliptin. The role played by these drugs in the adipose tissue is not yet well studied. NPY is one of DPPIV substrates and is also produced and secreted in the adipose tissue, among other tissues [94, 102, 103, 135]. Previous studies demonstrated that DPPIV stimulates lipid accumulation through cleavage of NPY_{1-36} in NPY_{3-36}, which leads to activation of Y_2 receptor (Ana P Marques; Joana Rosmaninho-Salgado, unpublished data). The mechanism by which NPY stimulates lipid accumulation is not yet known, nor the effects of gliptins on NPY-induced lipid accumulation.

Therefore, to understand the role played by gliptins on the adipose tissue, the main goals of this study are:

1) To evaluate the effect of gliptins on lipid accumulation, using a 3T3-L1 murine cell line
2) To investigate whether gliptins can modulate lipolysis or adipogenesis
3) To study the intracellular mechanism of action of gliptins
4) To investigate the effect of gliptins on NPY-induced lipid accumulation and NPY's mechanism of action

Chapter 2: Materials and Methods

2.1 Material:

3T3-L1 pre-adipocytes were obtained from the American type Culture Collection – LGC Promochem (Barcelona, Spain); Visceral and Epididymal Human pre-adipocytes cells and also the Lipolysis Assay Kit were obtained from Zenbio, Inc.; Dubeccos Modified Medium high glucose was obtained from Gibco (Barcelona, Spain); Cell plates were obtained from Orange Scientific (Geneva, Switzerland); Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, Oil red-O staining and isopropanol were obtained from Sigma (St Louis, MO, USA). NPY was obtained from Tocris Bioscience (Bristol, United Kingdom). BCA protein assay kit was obtained from Thermo Scientific Pierce. Antibody anti-PPARγ is from Santa Cruz Biotechnology (Heidelberg, Germany) and antibody anti-perilipin is from Cell Signaling, Inc. (Danvers, MA, USA). Hoechst 33342, antibody anti-rabbit IgG labelled with Alexa 488 were purchased from Molecular Probes (Invitrogen, Paisley, UK). rDPPIV and Vildagliptin (Galvus) were gently provided by Dr. Eric Grouzmann (Lausanne, Switzerlan). Sitagliptin (Januvia) was bought from Merck, and Saxagliptin (Onglyza) from Bristol-Myers Squib and Astra Zeneca.

2.2 Methods:

2.2.1 Cell culture

The murine pre-adipocyte cell line (3T3-L1) was plated in 22.1 cm^2 flasks and maintained in a humidified atmosphere of 5 % CO_2-95 % air. Cells were grown in Dubeccos Modified Medium (DMEM-F12) high glucose with phenol red and supplemented with 2.5 mM l-glutamine, 4.5 g/L glucose, 1.5 g/L $NaHCO_3$, 10% heat-inactivated fetal bovine serum (FBS) (45ºC, 30min.), 100U/mL penicillin, 100U/mL streptomycin and 0.25 µg/mL amphotericin B. At 80 % confluence, cell culture was splited 1:10 by incubating cells with trypsin solution (37ºC, 3min.) and subcultured in 22.1 cm^2 polystyrene culture plates.

2.2.2 Cell differentiation conditions

Pre-adipocytes were plated in 24-well plates (25 000 cells/well) and in 12-well plates (50 000 cells/well), until they reach confluence (day 0). After 2 days, the medium was removed and replaced by DMEM supplemented with a differentiation cocktail: IBMX (0.5 mM) and dexamethasone (0.25 µM) (day 2). After 3 days (day 5), the differentiation cocktail was removed and the culture medium changed to DMEM-HG. Every 2 days the medium was renewed until day 9. Cell incubation with insulin (1 µg/mL) was considered as the positive control. To test the effect of the different drugs in cell differentiation and lipid accumulation, these were incubated with insulin, together with selective inhibitors of rDPPIV (vildagliptin, sitagliptin and saxagliptin); with the selective inhibitors (without

insulin); and also with rDPPIV, in the presence and in the absence of the selective inhibitors. The negative control was cells that were not treated with insulin nor any other drug.

2.2.3 Oil red-O staining

Nine days after the induction of pre-adipocyte differentiation, cells were washed twice with Phosphate buffered saline (PBS) buffer and fixed with p-formaldehyde (4% in PBS) for 30 min at room temperature. Cells were then washed twice with PBS and once with distilled water. Cells were stained with Oil red-O dye (6:4, 0.6 % Oil red-O dye in water) for one hour and washed three times with water. Finally, Oil red-O dye was dissolved in 200µL of isopropanol. Absorbance was measured at 450 and 570 nm.

2.2.4 Immunocytochemistry

Following fixation and permeabilization, nonspecific binding was blocked with 3% BSA. Cells were incubated with primary antibody, anti-PPARγ (1:500) or anti-Perilipin A (1:100), for 90 minutes, at room temperature. After washing with PBS, cells were incubated with secondary antibodies for 1hour (1:200, anti-rabbit conjugated with Alexa Fluor 488), at room temperature. All antibodies were prepared in blocking solution (3 % BSA). Nuclei were labelled with Hoechst 33342 (1 µg/mL) for 3 minutes. Coverslips were mounted on glass slides and visualised on a fluorescence microscope (Axioskop 2 Plus, Zeiss, Jena, Germany), where images were acquired with the Axiovision software; or using a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany).

2.2.5 Total Protein extracts and Quantification

Cells were placed on ice, rinsed twice with ice-cold PBS and then lysed with RIPA buffer (50 mM Tris-HCl pH=8, 150nM NaCl, 1 % Triton, 0.5 % SDC, 0.1 % SDS) containing 100 µM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 µg/mL quimostatin, 1 µg/mL leupeptin, 1 µg/mL antiparin, 5 µg/mL pepstatin A (CLAP) and 1 mM ortovanadate, pH=7.4. Lysates were centrifuged at 3300 x g for 10 minutes at 4ºC, and the supernatants collected.

Protein concentration was determined using the BCA protein assay, where bovine serum albumin (BSA) was used to generate the standard curve.

2.2.6 Western blotting

Proteins were denatured by adding to each sample 1/5 of its volume in 6x concentrated denaturing solution, followed by heating to 95ºC, for 5 minutes.
Proteins were loaded in a 4-10% discontinuous polyacrylamide gel, in the presence of SDS (SDS-PAGE), and then separated by electrophoresis. Protein concentration was mainly between 40 and 60 µg/µL, depending on the antibody being used. Following gel electrophoresis, proteins were electrophoretically transferred from the gel to a polyvinylidene fluoride membrane (90 minutes at 750 mA). Prior to the transfer, membranes were activated with methanol. When the transfer was completed, membranes were blocked in 5 % non-fat milk in 0.1 % Tween 20 (TBS-T), for 60 minutes.

Antibodies incubation was performed overnight (4ºC) with either rabbit polyclonal anti-PPARγ (1:500) or anti-Perilipin (1:500), in 1% (m/v) of non-fat milk in TBS-T. To normalize protein concentration, membranes were then incubated with anti-β-actin (1:20000). After incubation, membranes were firstly washed for 30 minutes in TBS-T, secondly incubated with an alkaline phosphatase-conjugated anti-rabbit (1:200) or anti-mouse (1:200), for 30 minutes at room temperature, and finally, after being washed for 30 minutes in TBS-T, immunorreactive detection was performed by chemifluorescence with ECF substrate. Fluorescence intensity was quantified by *Quantity One* (Biorad).

2.2.7 Lipolysis Assay Kit (Glycerol Quantification)

A standard curve was prepared one hour prior to the assay. Seven days after the initiation of the differentiation protocol, 25 µL of media was removed from each condition and added to a specific well in a 96 well plate. Glycerol Reagent A was then added to each well, containing either the media or the standards. Absorbance was measured at 570 nm, after 15 minutes incubation at room temperature.

Simultaneously, total protein extracts were made and quantified, as described above. All absorbances were then normalized with the protein amount obtained in each sample.

2.2.8 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Data was analysed using one-way analysis of variance (ANOVA) followed by Dunnet's test. A value of $p<0.05$ was considered significant. Prism 5 (GraphPad Software, San Diego, CA) was used for all statistical analysis.

Chapter 3: Results

3.1 The role of gliptins on lipid accumulation

3.1.1 The role of gliptins on DPPIV-induced lipid accumulation

Previous studies in our laboratory showed that DPPIV induces lipid accumulation (Ana P. Marques; Joana Rosmaninho-Salgado, unpublished data). To study the effect of gliptins on lipid accumulation induced by DPPIV, adipocytes were incubated with rDPPIV (50 µg/mL) in the presence or absence of vildagliptin (2 nM), sitagliptin (20 nM) or saxagliptin (1 nM) during 7 days. Lipid accumulation was analysed using the Oil red-O staining assay. All three gliptins reduced rDPPIV-induced lipid accumulation: vildagliptin reduced 17.3 ± 3.1%, sitagliptin 29.9 ± 4.3% and saxagliptin 25.5 ± 5.6%, respectively, compared to lipid accumulation induced by rDPPIV (figure 3.1).

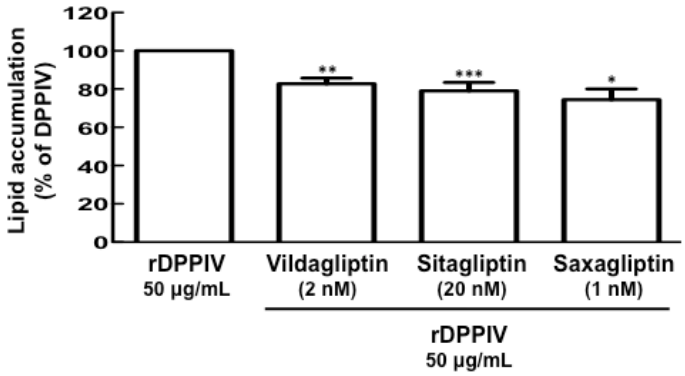

Figure 3.1. – Gliptins decrease rDPPIV-induced lipid accumulation.

Adipocytes were incubated with rDPPIV (50 µg/mL) in the presence or absence of the DPPIV selective inhibitors vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM) during 7 days. The Oilred O-staining assay was performed as described in Materials and Methods (see section 2). Results are expressed as the percentage of lipid accumulation compared to rDPPIV. Mean ± SEM, 3 to 7 different independent experiments, each condition performed in triplicate. *p<0.05 , ** p < 0.01 and ***p<0.001, compared to rDPPIV (50 µg/mL). One-way ANOVA was used as statistical test.

3.1.2 The role of gliptins on basal lipid accumulation

Differentiated adipocytes also express and release DPPIV [158]. To study the effect of gliptins have on basal lipid accumulation, adipocytes were incubated with vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM) during 7 days. Basal lipid accumulation was inhibited in the presence of gliptins: vildagliptin, sitagliptin, saxagliptin inhibited 29.4 ± 8.3%, 27.9 ± 8.4% and 21.5 ± 3.6%, respectively, compared to control (figure 3.2).

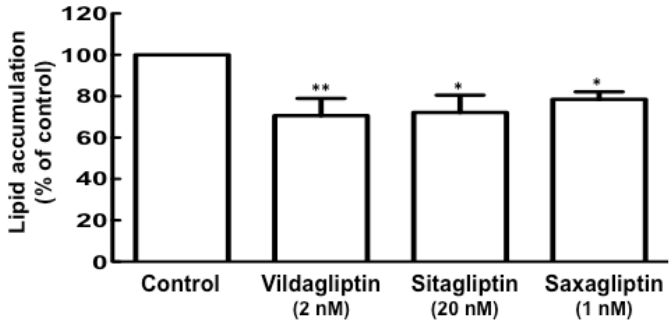

Figure 3.2. - The role of gliptins on basal lipid accumulation.

Adipocytes were incubated with the DPPIV inhibitors: vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM), for 7 days. The Oilred O-staining assay was performed as described in Materials and Methods (see section 2). Results are expressed as the percentage of lipid accumulation compared to control. Mean ± SEM, 6 to 7 different independent experiments, each condition performed in triplicate. *p<0.05 and ** p < 0.01, compared to control. One-way ANOVA was used as statistical test.

3.1.3 The role of gliptins on lipid accumulation induced by insulin

Insulin stimulates lipid accumulation, by preventing lipolysis [34] and by inducing lipogenesis [1]. To study the effect of gliptins on lipid accumulation induced by insulin, adipocytes were incubated with insulin (1 µg/mL) in the presence or absence of vildagliptin (2 nM), sitagliptin (20 nM), or saxagliptin (1 nM). All three gliptins decreased insulin-induced lipid accumulation: vildagliptin, sitagliptin and saxaglitin inhibited 22.4 ± 7.1%, 16.3 ± 3.5% and 23.8 ± 4.1%, respectively, compared to insulin (figure 3.3).

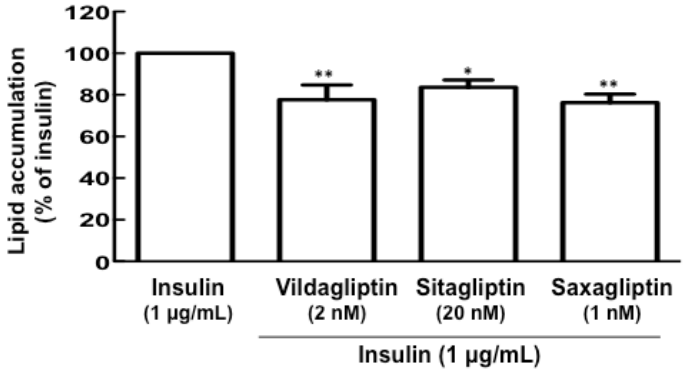

Figure 3.3. – Gliptins reduce insulin-stimulated lipid accumulation.

Adipocytes were incubated with insulin (1 µg/mL) in the presence or absence of the DPPIV selective inhibitors vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM) during 7 days. The Oilred O-staining assay was performed as described in Materials and Methods (see section 2). Results are expressed as the percentage of lipid accumulation compared to insulin. Mean ± SEM, 6 to 5 different independent experiments, each condition performed in triplicate. All values were normalized to 100% of insulin *p<0.05 and ** p < 0.01, compared to insulin (1 µg/mL). One-way ANOVA was used as statistical test.

3.2 Effect of gliptins on lipolysis

The effect of gliptins on lipid accumulation was also studied by analysing the effect of gliptins on glycerol release, which is a measure of lipolysis. We observed that vildagliptin, sitagliptin and saxagliptin did not affect basal glycerol release, when compared to control (figure 3.4).

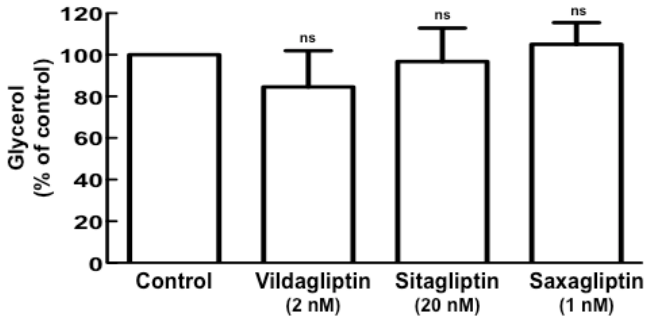

Figure 3.4. – Gliptins do not affect basal glycerol release.

Adipocytes were incubated with isoproterenol (1 µM), considered as a positive control, insulin (1 µg/mL) as the negative control and vildagliptin (2 nM), sitagliptin (20 nM) or with saxagliptin (1 nM), during 7 days The Lipolysis Assay kit was performed as described in Materials and Methods (see section 2). Results are expressed as the percentage of glycerol release compared to basal control. Mean ± SEM, 3 to 4 different independent experiments, each condition performed in triplicate. One-way ANOVA was used as statistical test.

Perilipin is a protein only expressed in differentiated adipocytes [36] and is present at the surface of lipid droplets and protects them against several lipases [35]. When lipolysis occurs perilipin levels decrease [38].

Adipocytes were incubated with vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM) during 7 days, and the perilipin levels were evaluated by Western Blotting assay. It was observed that gliptins do not affect perilipin levels (figure 3.5).

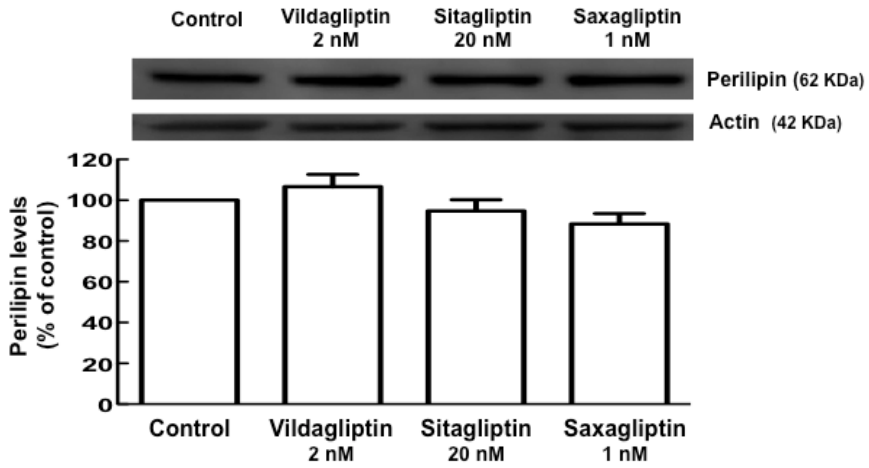

Figure 3.5. – Gliptins do not affect perilipin levels

Adipocytes were incubated with vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM) during 7 days. Whole cell extracts were performed and Western Blotting assay against perilipin (62 KDa) was performed as described in Materials and Methods (see section 2). Results are expressed as the percentage of perilipin levels, compared to control. Mean ± SEM, 8 different independent experiments, each condition performed in triplicate. One-way ANOVA was used as statistical test.

Before being degraded in lysosomes, perilipin leaves the surface of lipid droplets [38]. We evaluated the effect of gliptins on perilipin location on the surface of the lipid droplets of adipocyte by immunocytochemistry assay. Both in the presence or absence of gliptins, adipocytes showed perilipin (green) surrounding the surface of the lipid droplets (figure 3.6), suggesting that gliptins do not affect perilipin cell location.

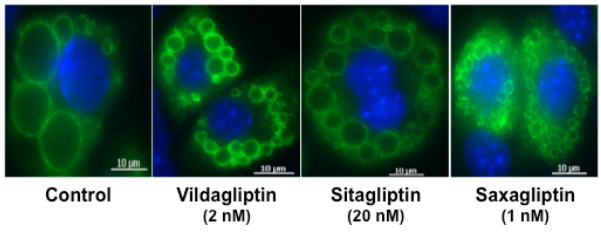

Figure 3.6. – Gliptins do not change perilipin location

Representative images of differentiated adipocytes, showing perilipin staining (green) on the surface of lipid droplets and in the cytoplasm. These cells were treated with vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM), during 7 days. Nuclei were labelled with Hoescht 33342 (blue). Immunocytochemistry was performed as described in Materials and Methods (see section 2). Representative images are shown.

The effect of DPPIV on lipolysis was also investigated by measuring glycerol release. Adipocytes were incubated with isoproterenol (1 μM, positive control) [33], insulin (1 μg/mL, negative control) [34] and recombinant DPPIV (rDPPIV, 50 μg/mL), during 7 days. Isoproterenol induced an

increase of 28.0 ± 3.0 % on glycerol release, and insulin decreased 45.3 ± 8.7%. rDPPIV did not change glycerol release (figure 3.7).

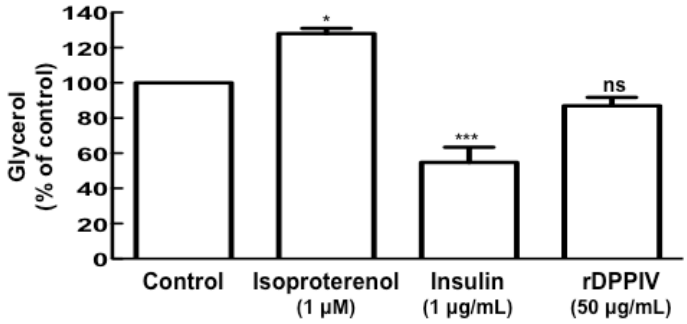

Figure 3.7. – DPPIV does not affect glycerol release.

Adipocytes were incubated with isoproterenol (1 µM, positive control), insulin (1 µg/mL, negative control) or with rDPPIV for 7 days. Glycerol release into the medium was quantified using a Lipolysis Assay Kit. The Lipolysis Assay kit was performed as described in Materials and Methods (see section 2). Results are expressed as the percentage of glycerol release compared to basal control. Mean ± SEM, 2 to 4 different independent experiments, each condition performed in triplicate. ***p<0.001, compared to control. One-way ANOVA was used as statistical test.

To evaluate the effect of DPPIV on perilipin levels, adipocytes were incubated with rDPPIV (50 µg/mL) and Western blotting assay was performed. We observed that rDPPIV does not affect perilipin levels, when compared to control (figure 3.8).

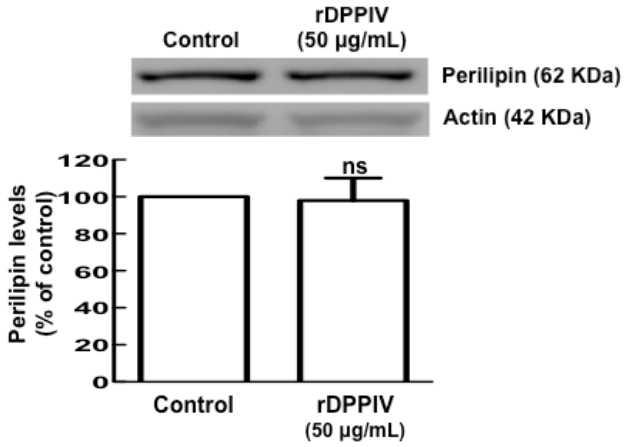

Figure 3.8. – DPPIV does not change perilipin levels.

Adipocytes were incubated with rDPPIV (50 µg/mL) during 7 days and whole cell extracts were performed. Western blotting assay was performed against the perilipin (62 KDa) immunoreactivity by Western Blotting, as described in Materials and Methods (see section 2). Results are expressed as the percentage of perilipin levels, compared to control. Mean ± SEM, 3 different independent experiments, each condition performed in triplicate. T-test was used as statistical test.

3.3 The role of gliptins on adipogenesis

In figures 3.4, 3.5 and 3.6 we showed that gliptins do not reduce lipid accumulation by inducing lipolysis. Since PPARγ is a transcription factor crucial for pre-adipocyte differentiation into adipocytes [18] we investigated the role gliptins on PPARγ levels. Adipocytes were incubated with vildagliptin (2 nM), sitagliptin (20 nM) or saxagliptin (1 nM) and PPARγ levels were determined by Western-bloting The three gliptins reduce PPARγ levels in 19.9 ± 5.9%, 15.0 ± 3.5% and 14.7 ± 6.5%, respectively (figure 3.9).

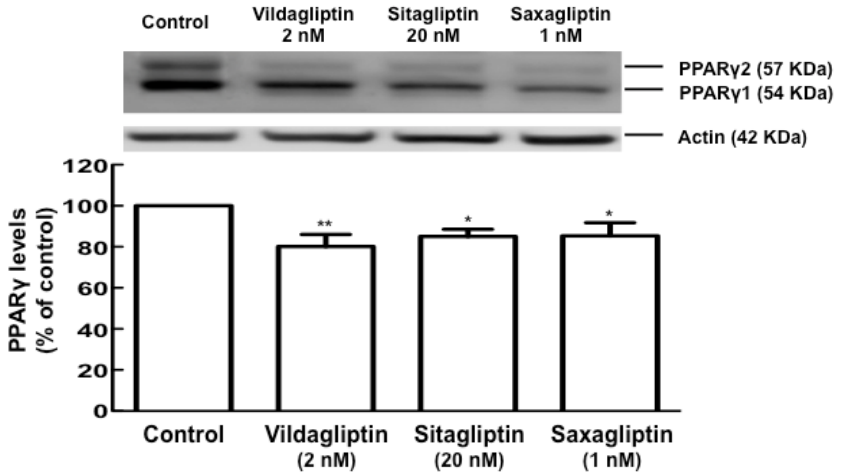

Figure 3.9 – Gliptins decrease PPARγ levels.

Adipocytes were incubated with vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM), for 7 days. Whole cell extracts were assayed for PPARγ immunoreactivity by Western Blotting, as described in Materials and Methods (see section 2). Results are expressed as the percentage of PPARγ levels, compared to control. Mean ± SEM, 7 to 14 different independent experiments, each condition performed in triplicate. *p<0.05 and ** p < 0.01 compared to control. One-way ANOVA was used as statistical test.

Differentiated adipocytes maintain PPARγ levels in order to keep the differentiated state [1]. To evaluate if gliptins could revert the differentiated state of adipocytes, by decreasing PPARγ levels [1], we determined PPARγ levels in cells previously treated with insulin (1 µg/mL) in the presence or absence of gliptins. The results show that PPARγ levels decrease 13.0 ± 3.3 %, 16.1 ± 2.6 % and 16.7 ± 6.5 % in the presence of vildagliptin (2 nM), sitagliptin (20 nM) or saxagliptin (1 nM), respectively (figure 3.10).

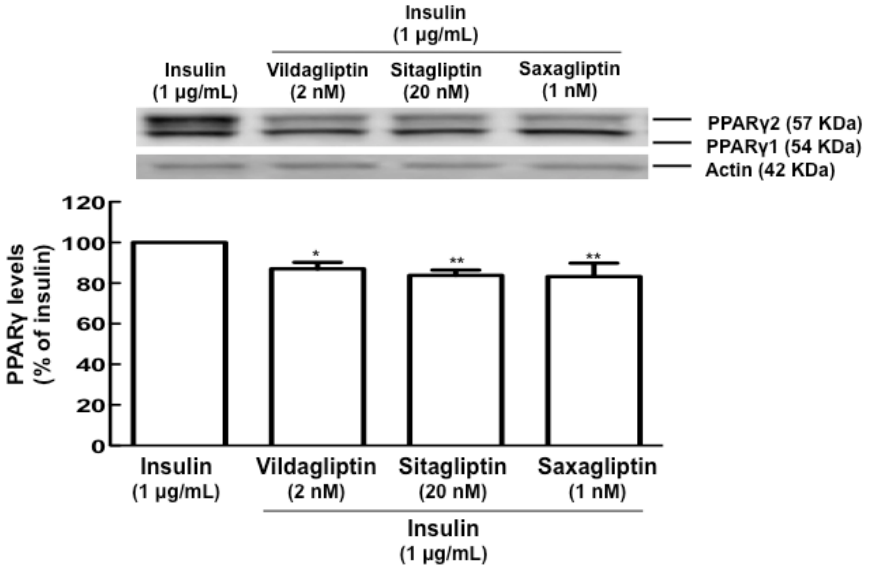

Figure 3.10. – Gliptins are reducing insulin induced PPARγ levels.

Adipocytes were incubated with insulin (1 µg/mL) in the presence or absence of vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM), for 7 days. Whole cell extracts were assayed for PPARγ immunoreactivity by Western Blotting, as described in Materials and Methods (see section 2). Results are expressed as the percentage of PPARγ levels, compared to insulin. Mean ± SEM, 5 to 9 different independent experiments, each condition performed in triplicate. *p<0.05 and ** p < 0.01, compared to insulin. One-way ANOVA was used as statistical test.

To further understand the mechanism of gliptins on inhibiting lipid accumulation, we investigated the involvement of PKA pathway on this effect. We incubated adipocytes with vildagliptin (2 nM), in the presence or absence of the PKA inhibitor (H89, 1 µM) during 7 days. Using Oil red-O staining assay, we quantified lipid accumulation and the results showed that in the presence of H89, lipid accumulation increased to values close to control (figure 3.11). We also incubated adipocytes alone with H89 and no basal effect was observed.

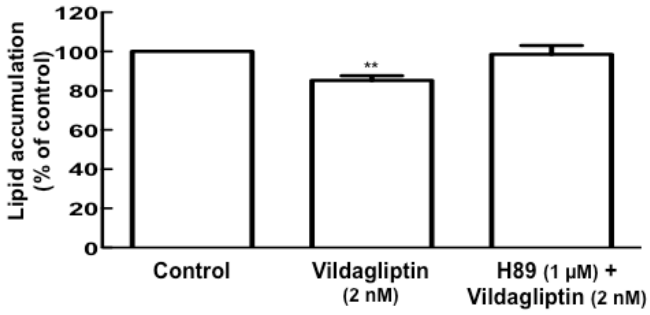

Figure 3.11 – Gliptins inhibit lipid accumulation through PKA.

Adipocytes were incubated with the vildagliptin (2 nM), in the presence or absence of the PKA inhibitor (H-89, 1 µM), during 7 days. The Oilred O-staining assay was used as described in Materials and Methods (see section 2). Results are expressed as the percentage of lipid accumulation compared to control. Mean ± SEM, 8 different independent experiments, each condition performed in triplicate. ** $p < 0.01$, compared to control. One-way ANOVA was used as statistical test.

Since gliptins also decrease lipid accumulation induced by insulin, we also tested PKA pathway in the presence of insulin (1 µg/mL) and vildagliptin (2 nM). The results show that when PKA inhibitor is present lipid accumulation increased to amounts near those induced by insulin (figure 3.12).

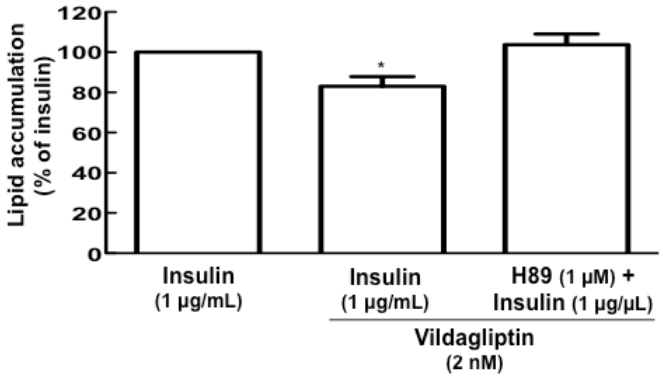

Figure 3.12. – Vildagliptin inhibits insulin-stimulated lipid accumulation through PKA.

Adipocytes were treated with insulin (1 µg/mL) and vildagliptin (2 nM), in the presence or absence of the PKA inhibitor (H-89,1 µM) during 7 days. The Oilred O-staining test was used as described in Materials and Methods (see section 2). Results are expressed as the percentage of lipid accumulation compared to insulin. Mean ± SEM, 5 different independent experiments, each condition performed in triplicate. *$p<0.05$, compared to insulin. One-way ANOVA was used as statistical test.

3.4 The role of gliptins on NPY-induced lipid accumulation

Previous work showed that DPPIV stimulates lipid accumulation by cleaving NPY in a NPY Y_2 receptor agonist, the NPY_{3-36} fragment (Ana P Marques; Joana Rosmaninho-Salgado, unpublished data). We further investigated the role of NPY on lipid accumulation induced by DPPIV by studying the effect of gliptins on lipid accumulation induced by NPY. The results show that, all three gliptins reduce NPY-induced lipid accumulation (fig 3.13). Vildagliptin, sitagliptin and saxagliptin decreased 12.8 ± 2.82 %, 32.9 ± 5.9 % and 24.9 ± 11.0 %, respectively, compared to NPY (figure 3.13).

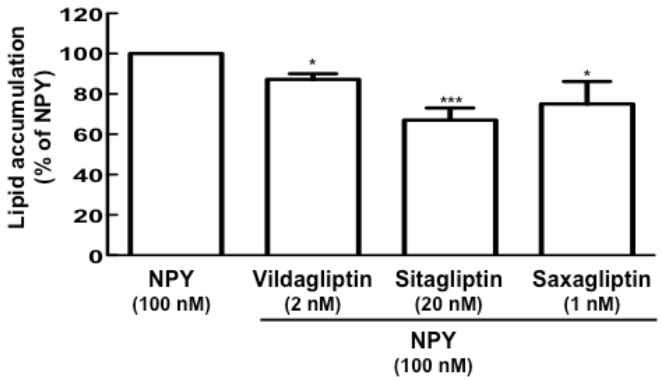

Figure 3.13. – Gliptins decrease NPY-induced lipid accumulation

Adipocytes were incubated with NPY (100 nM) in the presence or absence of vildagliptin (2 nM), sitagliptin (20 nM) and saxagliptin (1 nM) during 7 days. The Oil-red O-staining test was used, as described in Materials and Methods (see section 2). Results are expressed as the percentage of lipid accumulation compared to NPY. Mean ± SEM, 5 different independent experiments, each condition performed in triplicate. *p<0.05 and ** p < 0.01, compared to NPY. One-way ANOVA was used as statistical test.

We investigated the role of the PKA pathway on lipid accumulation induced by NPY. The results show that when NPY was incubated together with the PKA inhibitor H-89 (1 μM), the stimulatory effect of NPY on lipid accumulation was inhibited (figure 3.14).

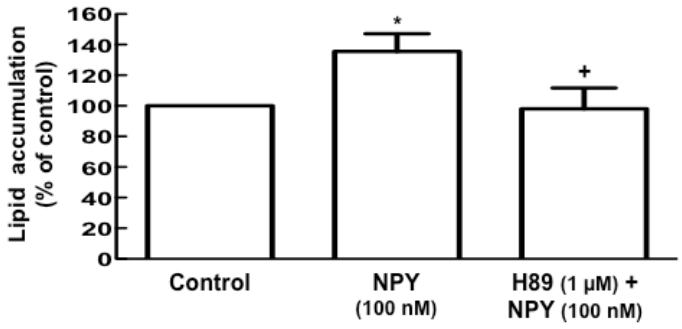

Figure 3.14. – NPY stimulates lipid accumulation through PKA.

Adipocytes were incubated with NPY (100 nM), in the presence or absence of H-89 (1 µM), for 7 days. The Oilred O-staining test was used as described in Materials and Methods (see section 2). Results are expressed as the percentage of lipid accumulation compared to control. Mean ± SEM, 7 different independent experiments, each condition performed in triplicate. *p<0.05, compared to control; +p<0.05 compared to NPY. One-way ANOVA was used as statistical test.

Chapter 4: Discussion

DPPIV selective inhibitors are the new class of drugs for the treatment of T2DM [61]. Until 2010, vildagliptin, sitagliptin, saxagliptin, were the only three gliptins commercialized in Europe and, except from vildagliptin, accepted by FDA. The present work is the first to suggest that these gliptins reduce lipid accumulation by inhibiting adipogenesis via PKA.

Using a 3T3-L1 cell line, we showed that gliptins reduce both basal and stimulated lipid accumulation in adipocytes. Moreover, unpublished data from our group demonstrated that DPPIV stimulates lipid accumulation and, with the present study it is also suggested that gliptins inhibit the stimulatory effect induced by DPPIV. In fact, our results are consistent with previous studies where type 2 diabetic (T2DM) mice treated with sitagliptin had lower fat depots and a decrease in adipocytes size [151]. It was also observed that C57BL/6 obese mice treated with sitagliptin for 12 weeks lost weight due to a decrease in adipose tissue [159]. Also, β-cell-specific glucokinase haploinsufficient (Gck +/-)) diabetic mice, under a high fat diet, became obese and with adipocyte hypertrophy. When des-fluo-sitagliptin was administered the hypertrophy was reduced [160], demonstrating the importance of DPPIV selective inhibitors on the adipose tissue.

Taking into account that gliptins have an important role on lipid accumulation, we studied the role of gliptins on lipolysis and adipogenesis. Our studies show that gliptins do not induce lipolysis (figures 4.1, 4.2 and 4.3). Vildagliptin, sitagliptin and saxagliptin, did not induce glycerol release to the medium, when compared to a basal situation. It is also described that when occurs a mobilization of perilipin from the surface of lipid droplets it indicates that lipolysis is occurring [38]. In our work we demonstrated that perilipin levels and its physical position around lipid droplets were similar in all conditions, indicating that gliptins are not involved in lipolysis. There is a study performed by others using different experimental approaches suggesting opposite results: vildagliptin given to T2DM patients increased post-prandial glycerol release in the adipose tissue [146].

We further investigated the role of gliptins on the inhibition of lipid accumulation. Several studies state that DPPIV plasma activity is increased in obese states [158, 165], and perilipin levels are decreased when compared to lean individuals and, consequently, lipolysis is increased [169-171]. So it would be expected that DPPIV induced lipolysis. However, our results show that DPPIV has no effect on perilipin levels or glycerol release and, consequently, no effect on lipolysis, (see figures 4.4 and 4.5.). On the other hand, the absence of effect of DPPIV on lipolysis is in agreement with previous results of our group which demonstrated that DPPIV induced not only lipid accumulation but also PPARγ expression (Ana P Marques; Joana Rosmaninho-Salgado, unpublished data). Other groups also observed the importance of DPPIV on adipose tissue formation. DPPIV knock-out mice and DPPIV deficient rats were resistant to obesity under high fat diets [162-164] and several studies state that DPPIV plasma activity is increased in obesity [158, 165]. In our work we also show that all the three gliptins were able to inhibit PPARγ expression induced by DPPIV (see figures 3.9 and 3.10). However, this role that gliptins have on PPARγ expression is contrary to the effect that other anti-diabetics have [172]. Thiazolidinediones (TZDs), for example, decrease insulin resistance by enhancing PPARγ expression, resulting in increased body weight [172]. On the contrary, gliptins act to increase the half-life of incretins and not to increase PPARγ expression [61, 155]. Several studies state that pre-adipocytes lacking the PPARγ gene are unable to differentiate into mature adipocytes and consequently, unable

to accumulate triglycerides [18]. In addition, when in differentiated adipocytes PPARγ expression is blocked, adipocytes dedifferentiate and lose the ability to accumulate lipid droplets [1]. Accordingly, it makes sense that gliptins reduce lipid accumulation by blocking the adipogenic process through inhibition of PPARγ expression. Therefore, we suggest that gliptins have an anti-adipogenic action on adipocytes leading to a decrease on lipid accumulation. This action may result in a decrease in total body weight as it was observed in mice that were under high fat diets and, with the administration of sitagliptin, lost weight [159, 160].

We further investigated the intracellular pathways involved on lipid accumulation modulated by gliptins. Our results show that gliptins action is through protein kinase A (PKA). Others have already showed that the protective action of sitagliptin against myocardial ischemia is via cAMP-dependent PKA activation [173]. Several studies demonstrated that PKA activation is necessary during the early stages of differentiation [24] but leads to inhibition of late stage of adipogenesis [25, 28, 29]. Furthermore, it was reported that mice continually expressing PKA had a reduction on the amount of adipose tissue and that these mice were resistant to diet-induced obesity [174]. The inhibitory role of PKA on adipogenesis was also demonstrated by using a PKA inhibitor in 3T3-L1 cells leading to a reduction on the time span needed for full adipogenesis [29]. All these results corroborate our hypothesis that gliptins lead to inhibition of adipogenesis by activating PKA.

NPY is a well-known substrate of DPPIV and it is also described that it increases lipid accumulation through stimulation of adipogenesis [94, 102, 103, 137] and inhibition of lipolysis [53, 94, 107]. In this study, we showed that gliptins decrease NPY-induced lipid accumulation. It is also described that in adipocytes, NPY decreases cAMP levels [30, 108] promoting adipogenesis [108]. In fact, in our study we showed that the stimulatory effect of NPY occurs through PKA pathway (figure 7.1.2). In addition, several studies demonstrated that NPY anti-lipolytic action is also mediated by PKA [37, 106, 108, 138]. Moreover, it was also demonstrated that NPY, in mouse chromaffin cells, modulates catecholamine release via PKA [175]. Also in a human neuroblastoma cell line SK-N-MC, NPY upregulates gene expression through modulation of cAMP levels and, consequently PKA activity [176, 177]. Other groups also studied the role of NPY on lipid accumulation. Some have suggested that NPY stimulates adipogenesis through NPY Y_1 receptor activation, both in primary rat adipocytes and in 3T3-L1 pre-adipocytes [103]. Others have showed that this effect is through NPY Y_2 receptor activation, in 3T3-L1 pre-adipocytes [178]. In addition, some of the unpublished data of our group showed that DPPIV stimulates adipocyte proliferation and lipid accumulation through activation of the Y_2 receptor. Here, we purpose that NPY stimulates lipid accumulation through Y_2 receptor in a PKA dependent manner.

In conclusion, in this study we demonstrate that vildagliptin, sitagliptin and saxagliptin have an anti-adipogenic effect via PKA, resulting in a decrease on lipid accumulation without lipolysis increase. In addition, NPY-stimulated lipid accumulation through Y_2 is dependent on PKA and this NPY effect is blocked by these three gliptins. These results suggest that gliptins can be used as new putative pharmacological strategies to prevent adipose tissue increase without the risk of dyslipidemia.

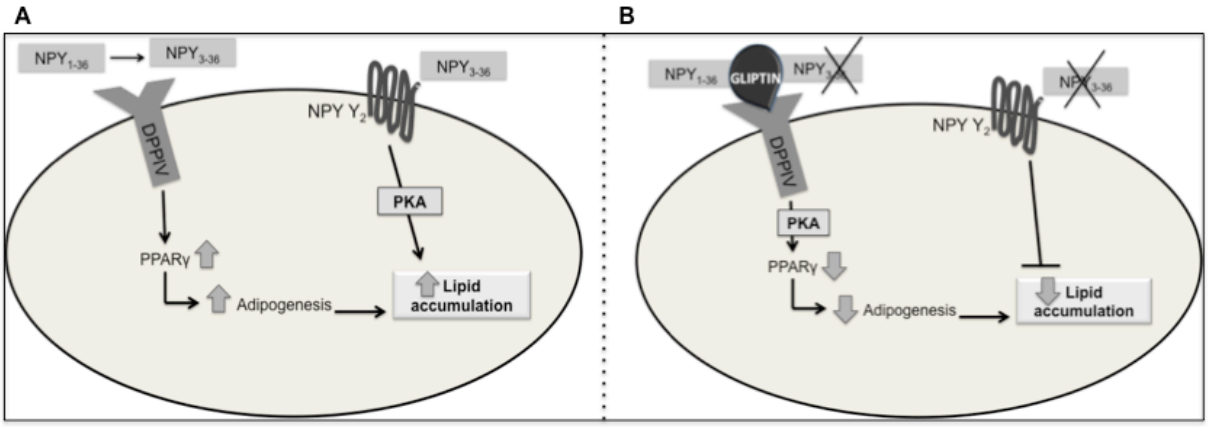

Figure 4.1 – Gliptins inhibition mechanism of DPPIV-induced lipid accumulation

A) DPPIV stimulates adipogenesis and, consequently lipid accumulation. To increase adipogenesis DPPIV increases PPARγ levels and, simultaneously cleaves NPY_{1-36} into NPY_{3-36}. The cleaved form of NPY activates the NPY Y_2 receptor that, by modulation of PKA, increases lipid accumulation. **B)** Gliptins reduce DPPIV-induced lipid accumulation is due to increased PKA activation that, consequently, leads to a decrease of PPARγ levels. This reduction blocks adipogenesis and decreases lipid accumulation. On the same time, DPPIV is no longer able to cleave NPY_{1-36} into NPY_{3-36}. The not cleaved form of NPY is not a NPY Y_2 agonist and, as a result, this receptor is not activated and lipid accumulation is not stimulated. Dipeptidyl-peptidase IV, DPPIV; peroxisome proliferator-activated receptor γ: PPAR γ; Neuropeptide Y: NPY; Protein kinase A: PKA; Neuropeptide Y Y_2 receptor: NPY Y_2.

Chapter 5: Conclusions

The results presented in this thesis suggest the following main conclusions:

1. Vildagliptin, sitagliptin and saxagliptin inhibit DPPIV-induced lipid accumulation;
2. Vildagliptin, sitagliptin and saxagliptin inhibit adipogenesis by decreasing PPARγ levels through activation of PKA pathway;
3. Vildagliptin, sitagliptin and saxagliptin do not induce lipolysis;
4. NPY-stimulated lipid accumulation occurs through PKA;
5. Vildagliptin, sitagliptin and saxagliptin inhibit NPY-stimulated lipid accumulation;

In summary, in this work we show that gliptins are able to inhibit adipogenesis, through activation of PKA pathway and that this inhibition leads to a decrease on lipid accumulation and has no effect on lipolysis. It was also observed that NPY-stimulated lipid accumulation is also through modulation of PKA and that this stimulus can be inhibit by gliptins. We suggest that DPPIV inhibitors may prevent the increase of adipose tissue without the risk of dyslipidemia.

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